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Development of Herpes Simplex Virus 1 vector for dendritic cell based immunotherapy of malaria

Thesis submitted to the University of London
for the degree of Doctor of Philosophy

by

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Abstract

Herpes simplex virus 1 (HSV-1) naturally infects dendritic cells but this prevents the cell from undergoing maturation. Removal of the virus host shutoff protein (vhs) has been shown to improve the ability of both human dendritic cells and mouse bone marrow dendritic cells to mature following virus infection. An HSV-1 vhs⁻ backbone was further modified by deletion of the ICP47 gene and mutation of the VP16 gene resulting in a vector that does not interfere at all with the ability of mouse dendritic cells to mature. This virus therefore, provided a good candidate for use as vector for antigen delivery in immunotherapy. A recombinant vhs⁻ICP47⁻VP16⁻ virus expressing the full length influenza nucleoprotein (NP) was constructed and used to determine the ability of this virus to induce immune responses upon immunisation with virus alone or upon immunisation with infected DCs. Both cellular and humoral responses were investigated and a CD8⁺ CTL response to both the NP gene and to the virus was obtained after different immunisation regimes confirming the potential of the virus to be used for immunotherapy.

In the attempt to further improve the vector, mGMCSF was added to the viral constructs and viruses expressing both mGMCSF and NP or full length plasmodium *yoelii* sporozoite surface protein 2 (PySSP2) or circumsporozoite protein (PyCS) were constructed. The effect of co-expressing mGMCSF from the virus was investigated. A convincing CD8⁺ CTL response was obtained against the antigen upon direct virus injection though co-expression of mGMCSF did not seem to confer a significant advantage.

Overall an HSV-1 vector has been developed and optimised for the infection of dendritic cells and then successfully used for induction of specific CTL and antibody response to full length NP, PySSP or PyCS.

Publications

Samady, L., **Costigliola, E.**, MacCormac, L., McGrath, Y., Cleverley, S., Lilley, C. E., Smith, J., Latchman, D. S., Chain, B. & Coffin, R. S. (2003). "Deletion of the virion host shutoff protein (vhs) from herpes simplex virus (HSV) relieves the viral block to dendritic cell activation: potential of vhs- HSV vectors for dendritic cell-mediated immunotherapy". *J Virol* 77, 3768-76.

Abstracts

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Chapter 1

Introduction

1.1 Malaria

1.1.1 Malaria epidemiology

Malaria is still one of the major cause of mortality and morbidity world-wide, accounting for 1-3 million deaths, mainly in children under the age of 5, with 300-500 million new cases each year (WHO 1999), mostly in sub-Saharan Africa. Malaria is caused by the *Plasmodium* parasite, which is transmitted by the bite of an infected female *Anopheles* mosquito. The four strains that can infect humans are *plasmodium falciparum*, *vivax*, *malariae*, and *ovale*, distributed differently across the endemic countries. *P. falciparum* is predominant in Africa where it accounts for 90% of infections and causes the most severe form of the disease. *P. falciparum* infection does not relapse but can cause chronic infections and be fatal. *P. vivax* is mainly found in Asia, South America, and Oceania, and while causing clinical malaria, is rarely fatal and in African populations is mainly restricted to individuals negative for the Duffy antigen. Finally *P. malariae* and *ovale* cause infrequent cases of mild malaria especially in Africa. *P. ovale* in particular may be found in mixed infections with other species.

The clinical symptoms of malaria usually include fever, anaemia, metabolic acidosis, impaired circulation, hypoxia and cerebral malaria. These are due to infected erythrocytes that bind to endothelial cells eventually leading to obstruction of microvasculature in tissues and organs.

1.1.2 The malaria life cycle

Malaria has a complicated, multistage life cycle that makes construction of a vaccine challenging. The bite of the infected *Anopheles* injects sporozoites into the bloodstream of the host. These quickly reach the liver (within 30min) and infect hepatocytes where they replicate forming exo-erythrocytic shizonts that contain as many as 30,000 merozoites, which are released by cell rupture. These merozoites released into the bloodstream have surface antigens that allow binding and infection of erythrocytes, initiating the asexual reproductive cycle responsible for the symptoms of clinical malaria. Within 48hr, the merozoites mature and replicate in the red blood cells forming schizonts that contain as many as 36 merozoites each. Rupture of the erythrocytes releases new merozoites that in turn

infect more red blood cells. Some merozoites develop into female or male gametocytes, which are then taken up by the mosquito in a subsequent blood meal. Inside the mosquito, the sexual replication cycle takes place by fusion of the gametocytes, developing into an oocyst in the mosquito gut. The sporozoites that are released by the oocyst then migrate to the salivary gland from where a new cycle begins (figure 1.1).

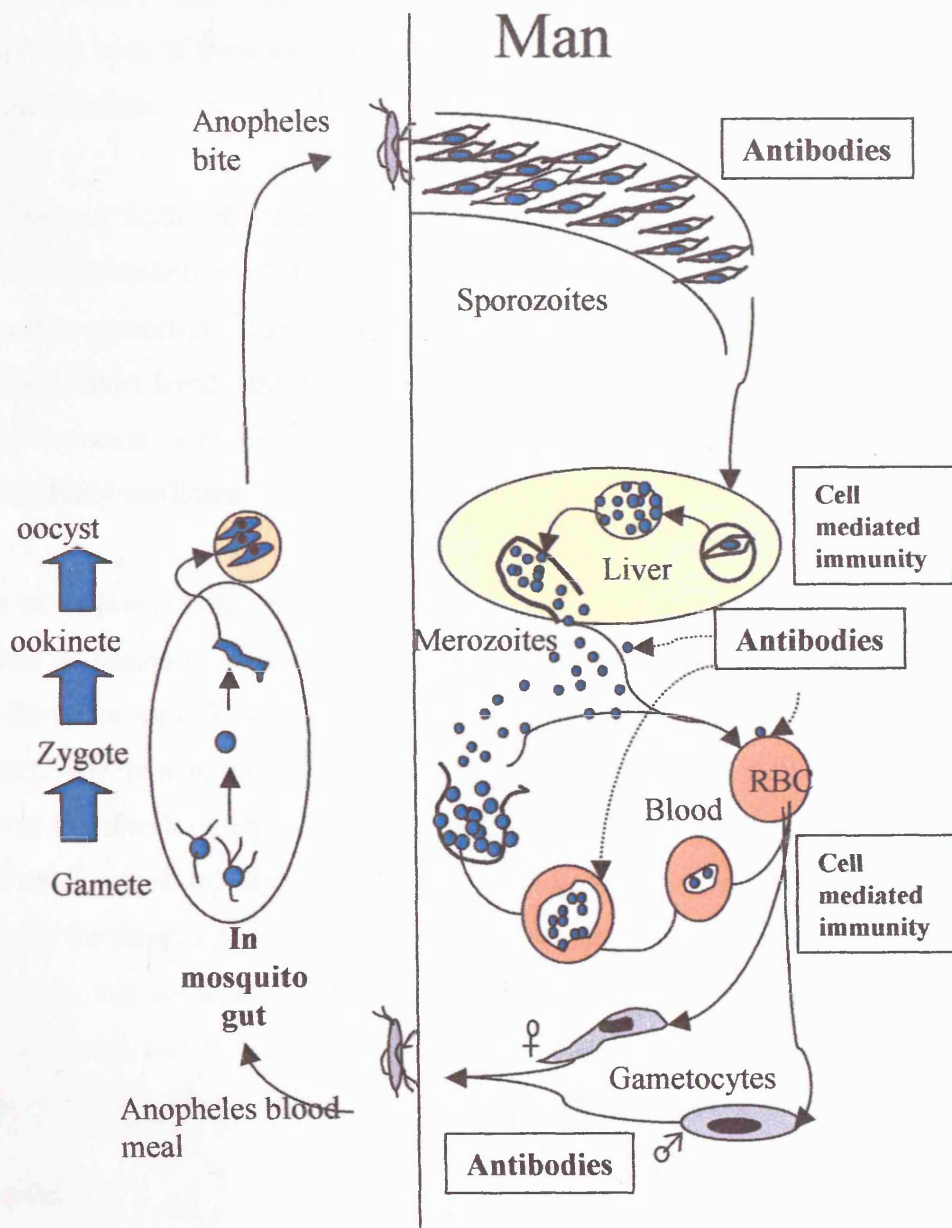


Figure 1.1: The malaria life cycle

Schematic representation of the stages of malaria life cycle with the type of immune response generated at each stage.

1.1.3 Protective immune responses to malaria

The malaria parasite interacts with each arm of the immune system stimulating various responses and also modulating responses to avoid clearance. Understanding both of these areas is therefore important for the construction of an appropriate vaccine.

The presence of both intracellular and extracellular stages in malaria infection implies that stimulation of both cellular and humoral responses would be necessary for protection (figure 1.1). Natural exposure to malaria induces, in human hosts, short lived, strain specific immunity (McGregor, 1974). Therefore, continued exposure is required to maintain immunity. This immunity is both T cell and antibody mediated.

The type of response induced by malaria infection has mainly been investigated by analysis of naturally immune individuals, the responses induced in infected mice, or the serum of volunteers or mice that have been immunised with irradiated sporozoites. The two main stages of the malaria life cycle that have attracted attention in the development of vaccines are the pre-erythrocytic liver stage and the blood stage, involving distinct types of responses. Cellular immunity seems to be necessary for targeting the liver stage to stop infection by blocking the release of merozoites. An immune response against the blood stage of the parasite is equally important, aimed at blocking red blood cell invasion, inhibit parasite growth or interfere with cytoadherence of infected erythrocytes.

CD8⁺T cells:

Cytotoxic T cells and IFN γ producing cells can induce elimination of intracellular parasites. Several lines of evidence have identified these as the major players in liver stage immunity.

Naturally immune individuals have CTLs specific for the pre-erythrocytic stage antigens (Aidoo et al., 1995, Hill et al., 1992) , and elimination of these cells can reverse the protective effect (Schofield et al., 1987, Weiss et al., 1988). Semi-immune individuals and children with malaria, on the other hand, only have low levels of CTL (Aidoo et al., 1995).

The important role played by CD8⁺T cells in the immune response to malaria was shown initially by analysing the response induced by immunisation with irradiated sporozoites. Mice receiving irradiated *P. yoelii* or *P. berghei* sporozoites were protected against subsequent challenges with sporozoites, and CD8⁺T cells against the circumsporozoite surface protein (CS) (Rodrigues et al., 1991, Romero et al., 1989, Weiss et al., 1992) and sporozoites surface protein 2 (SSP2) (Wizel et al., 1995) were identified and shown to recognise and destroy intrahepatic parasites (Romero et al., 1989, Schofield et al., 1987). Similarly, volunteers receiving irradiated *P. falciparum* could produce CS (Malik et al., 1991) and SSP2 (Wizel et al., 1995) specific CD8⁺T cells. Depletion experiments further confirmed the importance of these cells by abrogating the immunity induced by irradiated sporozoites (Schofield et al., 1987, Weiss et al., 1988).

Insights into the possible protective role of CD8⁺T cells also came from adoptive transfer of the cloned CS (Rodrigues et al., 1991, Romero et al., 1989, Weiss et al., 1992) and SSP2 (Khusmith et al., 1991) specific CD8⁺T cells into naïve mice. These protected mice against liver stage development upon sporozoite (but not infected erythrocyte) challenge. Similarly, mice receiving tumour cells expressing SSP2 showed CD8⁺T cell mediated protection against parasite challenge (Khusmith et al., 1991).

Interestingly, CD8⁺T cells require a ~24hr “induction” period to become fully active to eliminate infected hepatocytes. After this initial phase the T cells follow a very tight program of action, undergoing maximal expansion between 3-4 days from activation, followed by a dramatic reduction in cell number between days 4-7, and finally reaching stabilisation and production of memory T cells (Tsuji & Zavala, 2003). CD8⁺T cells mainly respond by releasing IFN γ , and this cytokine in particular (also released by CD4⁺T cells) has been associated with the protective immunity to malaria, making it important also to investigate the role of CD4⁺T cells (Doolan & Hoffman, 2000, Moreno et al., 1991, Schofield et al., 1987, Seguin et al., 1994).

CD4⁺T cells:

In an anti-malaria immune response, CD4⁺T cells are as important as CD8⁺T cells in that their absence results in a more than 90% reduction in CD8⁺T cell activity as they are in fact required for the survival of the newly activated CD8⁺T cells (Carvalho et al., 2002).

CD4⁺T cells have been shown to be present and specific for both pre-erythrocytic and erythrocytic stage antigens (Aidoo et al., 1995, Good, 1988, Hill et al., 1992, Malik et al., 1991, Nardin & Nussenzweig, 1993). Th1 responses are required both during initial infection to promote a cell-mediated CD8⁺T cell response at the liver stage, as well as during the blood stage. CD4 Th2 cells, on the other hand, seem to be necessary to control parasitaemia after the first cycle of infection, during the blood stage, to promote neutralising antibody responses (Molano et al., 2000, Su & Stevenson, 2002).

In addition to their role as helper cells for CD8⁺T cells and antibodies, CD4⁺T cells have also been shown to act directly against the malaria liver stage. Transfer of CD4⁺ Th1 or Th2 cell clones from mice immunised with irradiated sporozoites (Tsuiji et al., 1990) or with various synthetic peptides representing different epitopes of PyCS (Renia et al., 1993, Takita-Sonoda et al., 1996), into naïve mice could in fact confer protection in the first case, and inhibit liver stage parasitaemia in the second.

CD4⁺T cells may also play a role in the clearance of blood stage parasites, both CD4⁺T cell clones and polyclonal populations of T cells can adoptively transfer protection, apparently in the absence of antibodies (Brake et al., 1988); (Amante & Good, 1997). Nonetheless, in humans, better evidence exists for antibody-mediated immunity being the main effective response in blood stage infection (Good et al., 1998).

Finally, deficiency of CD4⁺T cells in mice, showed that under these circumstances, the immunity stimulated by the irradiated sporozoites is abrogated (Rodrigues et al., 1993), and the protective response induced by immunisation

with PySSP2 (Wang et al., 1996) or PyHEP17 (Charoenvit et al., 1999) synthetic peptides is prevented.

$\gamma\delta$ T cells:

$\gamma\delta$ T cells are activated by binding to a schizont-associated antigen (Pichyangkul et al., 1997) but also require exogenous cytokines (Elloso et al., 1994, Waterfall et al., 1998). They expand early in infection with *P. falciparum* (Hviid et al., 2001, Langhorne et al., 1994) and *P. vivax* (Langhorne et al., 1994), including primary infection, and produce large amounts of IFN γ (Hensmann & Kwiatkowski, 2001, Hviid et al., 2001) before the activation of other antigen specific T cells (Choudhury et al., 2000). $\gamma\delta$ T cells are involved in liver stage immunity (Schmieg et al., 2003), but are not essential for the resolution of infection (Stevenson & Riley, 2004). In the absence of $\gamma\delta$ T cells, mice immunised with irradiated sporozoites show a reduced protective immunity (Tsuji et al., 1994), an increased parasitaemia in the liver (McKenna et al., 2000), as well as a few days delay in the elimination of parasites (Langhorne et al., 2002). Adoptive transfer of these cells from mice immunised with irradiated sporozoites into naïve mice, results in reduced liver stage infection in the recipient mice after sporozoite challenge (Tsuji et al., 1994).

NK cells:

NK cells are mainly seen as effectors against the blood stage of the disease. They are not seen in all immune individuals, but appear to be the first cells to react upon in vitro exposure of human PBMCs to *P. falciparum* infected erythrocytes (Artavanis-Tsakonas & Riley, 2002).

Upon infection, NK cells release IFN γ , an essential step for the development of protective immunity to malaria which is associated with the clearance of infection (De Souza et al., 1997, Mohan et al., 1997). Absence of NK cells induces a more rapid increase in blood parasitaemia and less efficient disease resolution following primary infection (Mohan et al., 1997). A failure of NK, and possibly $\gamma\delta$ T cells, to produce IFN γ early on, results in lethal infection (De Souza et al., 1997, Mohan et al., 1997). Although IFN γ is released from NK cells in an IL12 dependent way,

both IL12 and IL18 are not sufficient for activation of NK cells as direct contact with the infected erythrocytes is also required (Artavanis-Tsakonas & Riley, 2002).

Antibody responses:

Antibodies are the main response against the erythrocytic stage of malaria and are directed against the merozoite molecules required for invasion of erythrocytes or aimed at stimulating antibody-dependent cytotoxicity (Druilhe & Perignon, 1994, Good & Doolan, 1999).

Sera from people living in endemic areas contain antibodies against merozoite antigens that prevent red cell invasion (Bull & Marsh, 2002). Anti-MSP1 (merozoite surface protein-1) antibodies in particular, found in the sera of immune individuals, can mediate inhibition of parasite infectivity in vitro (Egan et al., 1999) and have been associated with resistance to clinical malaria (Egan et al., 1996). In addition, immune individuals have high titres of antibodies against the erythrocyte membrane protein (PfEMP1) to inhibit binding to endothelial cells (Miller et al., 2002), and studies carried out in monkeys show that the level of protection correlates with the level of anti-PfEMP1 antibodies (Baruch et al., 2002). Purified immunoglobulins from naturally immune individuals transferred into partially immune children can clear the parasite in vivo, but not in vitro, and this has been suggested to rely on anti-PfEMP1 antibodies (Mahanty et al., 2003, McGregor, 1964).

Immunisation with irradiated sporozoites can also induce a humoral response as well, targeted at the sporozoite surface proteins. These antibodies can effectively inhibit parasite infectivity in vitro (Nardin et al., 1982, Zavala et al., 1985), and passive transfer protected naïve mice and macaques against viable sporozoite challenge (Charoenvit et al., 1991, Potocnjak et al., 1980). Nonetheless, pre-erythrocytic immunity is mainly a cellular response, whereas antibodies are most important at the erythrocytic stage.

Macrophages:

Macrophages have the ability to phagocytose infected erythrocytes in the absence of appropriate antibodies (Doolan et al., 2003, Good & Doolan, 1999, Stevenson & Riley, 2004), or to act as effector cells mediating antibody-dependent cellular inhibition (Stevenson & Riley, 2004). Macrophages are activated by IFN γ and TNF α released by other cells and function by producing anti-parasite molecules such as nitric oxide to eliminate infection.

Cytokines:

Malaria infection emerging from the liver is co-ordinated with release of IFN γ , TNF, IL12 and IL8 to fight infection in the serum of infected individuals, or in vitro (Stevenson & Riley, 2004). IFN γ is the main effector cytokine in malaria responses, stimulated by irradiated sporozoites and released by CD8⁺T, CD4⁺T, $\gamma\delta$ T and NK cells. IFN γ can probably induce infected hepatocytes to release nitric oxide leading to the arrest of the parasite development (Doolan & Hoffman, 2000) although there is no in vivo evidence that malaria antigens are presented on the surface of hepatocytes (Krzych et al., 2000).

Although IFN γ and TNF α are both associated with parasite clearance, they are also associated with severe malaria if their levels become toxic (Dodoo et al., 2002, Harpaz et al., 1992, Kwiatkowski et al., 1990). TGF β and IL10 are immunoregulatory cytokines in infection in both mice (Li et al., 2003, Omer et al., 2003) and humans (Dodoo et al., 2002, Kurtzhals et al., 1998) in that they balance the level of release of IFN γ and TNF α .

1.1.4 Immune evasion by plasmodium

Having such a complicated life cycle that includes numerous replication cycles at various locations in the host, is itself a mechanism for the parasite to avoid immune responses. In addition, the plasmodium has other immune evasion mechanisms that include frequent antigenic variation (Newbold, 1999, Plebanski et al., 2002), altered peptide ligand (APL) antagonism (Plebanski et al., 2002), and specific interactions with lymphocytes that result in inhibition of immune responses.

Antigenic variation:

Antigenic variation is a mechanism frequently used by parasites to escape immune responses. Some key plasmodium antigens display antigenic variation and although there are some conserved peptides within the protein, it is very difficult to include them in any vaccine. The plasmodium genome contains variant genes that have been grouped into “variant gene families”. For the *P. falciparum* *var*, *rif*, *Stevor* are the three main families (Rasti et al., 2004). Each family includes the different copies of one gene scattered around the genome. PfEMP1 encoded by the *var* family of roughly 60 genes, has been the most widely studied. PfEMP1 is the main antigen responsible for adherence to endothelial cells, and would therefore be a strong candidate as a vaccine target. However, since a different gene is expressed in each generation, it is very difficult to generate a targeted immune response (Saul, 1999). The high degree of variability, rapid rate of variation and high copy number of these genes within each parasite therefore limits the effectiveness of stimulating an immune response against this protein.

Altered peptide ligands (APL):

The use of altered peptide ligands by the parasite consists of presenting variants of the immunogenic epitopes on the surface of the cell. Instead of activating the T cell these epitopes antagonise the T cell receptor preventing effector functions such as cytotoxicity, cytokine release and proliferation or priming memory T cells from naïve precursors (Plebanski et al., 1999a, Plebanski et al., 1999b).

The CS protein, towards which many vaccines are targeted, itself contains highly polymorphic T cell epitopes. Fourteen variants of the immunodominant CD4⁺T cell epitope have been identified (Good, 1988), and three out of the four known human CTL epitopes, are polymorphic (Aidoo et al., 1995).

Naturally occurring altered peptide ligands (APL) variants have been shown to inhibit proliferation and IFN γ production from T cells, but the mechanism of action may vary depending on the variation. A CS APL has been shown to inhibit IFN γ release by promoting the switch towards IL10 production which can happen either when the epitope is co-presented on the same cell as the immunostimulating epitope or on a separate antigen presenting cell (Plebanski et al., 1999a). A different APL for the same epitope was shown not to have any effect if expressed on a different cell suggesting that various mechanisms are involved (Plebanski et al., 1999a).

Inhibition of lymphocyte action:

Although it has been possible to study the protective immune responses to the liver stage of malaria by infecting mice with irradiated sporozoites, this does not reflect the natural immune response pattern. Humans or mice receiving infectious sporozoites show a very small number of T cells reactive to the liver stage, and increased levels of IL10. The parasite has therefore developed mechanisms to inhibit T cell activity.

Inhibition of CD8⁺T cells by malaria infection has been reported in both mice (Ocana-Morgner et al., 2003) and humans (Good & Doolan, 1999, Urban et al., 1999). T cells are inhibited indirectly, by impairment of dendritic cell activity. Although it is the immune response to the liver stage that is low, DC activity is not inhibited by the sporozoites. Instead, studies in both humans (Ocana-Morgner et al., 2003, Urban et al., 1999, Urban & Roberts, 2003) and mice (Ocana-Morgner et al., 2003) using *P. falciparum* or *yoelii* infected erythrocytes, have shown that it is this interaction of erythrocytes with DCs that prevents cell maturation, thus activation of pre-erythrocytic T cells (Ocana-Morgner et al., 2003).

During the course of parasitaemia, infected erythrocytes interact with DCs possibly by binding to CD36 and CD51 on human PBMC (Urban et al., 2001). This interaction interferes with the Toll like receptor 4 (TLR4) activation mechanism, responsible for normal LPS induced cell maturation and for the secretion of IL12, also seen in macrophages, resulting in reduced TNF α and IL1 secretion during malaria infection (Schwarzer et al., 1992). As a consequence of this interaction, dendritic cells are driven to secrete IL10 rather than IL12, previous liver stage CD8 responses induced by irradiated sporozoites are reduced, and new anti-liver CD8 responses are prevented. Interestingly, despite the known effect of IL10 on inhibiting Th1 responses, the lack of T cell responses does not seem to be due to the presence of this cytokine but rather to the decreased level of IL12 or to other soluble molecules released by the cells (Ocana-Morgner et al., 2003). Additionally, although it has recently been shown that even intact uninfected erythrocytes can specifically inhibit phagocytosis of infected red blood cells by peripheral blood monocytes (Struik et al., 2004), the reduced level of T cell stimulation does not seem to be due to problems in the internalisation of the antigen by the DCs either (Ocana-Morgner et al., 2003).

Following from these effects, because the DCs that are in circulation after an initial blood stage infection are prevented from undergoing maturation, they can persist for longer, thus ensuring long term inhibition of T cell responses, even in the presence of only small amounts of infected erythrocytes (Ocana-Morgner et al., 2003).

Despite the evidence to support this immune evasion mechanism, there is controversial evidence showing that interaction between *P. yoelii* (Luyendyk et al., 2002, Perry et al., 2004) or *P. chabaudi* (Seixas et al., 2001) infected erythrocytes and BMDC still allows upregulation of maturation markers on the surface of the DC followed by activation of CD8⁺T cells with release of TNF α , IL12, and IFN γ (Seixas et al., 2001), though in the first case T cell proliferation and IL12 secretion were inhibited.

1.1.5 Malaria vaccines

1.1.5.1 The ideal vaccine

A vaccine should aim to induce long term immunity, especially for malaria since naturally exposed individuals gain only short term immunity that requires constant re-exposure. The aim of a malaria vaccine is to induce both cellular and humoral immune responses to multiple antigens covering the various stages of infection. The immune response needs to be greater than the one observed in naturally immunised individuals, and non-strain specific.

Whether T cell or B cell immunity should be stimulated depends on the stage of the disease targeted (Figure 1.2). As soon as the sporozoites are injected, neutralising antibodies should be present to inhibit hepatocyte invasion, but once in the liver, cell mediated immunity involving CD4⁺T cells and CD8⁺ cytotoxic T lymphocytes as well as various cytokines are required to kill infected hepatocytes and inhibit intrahepatic parasite growth. A pre-erythrocytic vaccine would be useful in stopping the parasite from reaching the blood cells, but the disadvantage with this is that no natural immunity would actually be stimulated, which would be useful for travellers but less useful for populations living in endemic areas.

To target the erythrocytic stage, antibodies against the merozoites are appropriate to inhibit invasion of red blood cells, to enable antibody mediated elimination of infected erythrocytes as well as inhibiting adhesion of erythrocytes to host cells. Cytokine mediated elimination of infected erythrocytes would also be useful at this stage of infection. The major targets of neutralising immune responses are the merozoites or sporozoites receptors that mediate invasion of RBCs and hepatocytes (Miller et al., 2002). These are found on the cell surface, or in organelles (micronemes, and in rhoptries) present on the invasive end of sporozoites and merozoites, but since RBCs lack MHCI and MHCII, it is only after cell rupture at the ring stage that the parasite is exposed to antibodies. Immunity targeted at the blood stage antigens does not achieve complete parasite clearance (Hoffman et al., 1987). A vaccine targeting this stage would therefore need to inhibit cell invasion as well as parasite growth and replication, thus

avoiding the actual symptoms of clinical malaria while stimulating the building of natural immunity. This would be ideal for endemic populations.

Finally, the sexual stage should be targeted by antibodies, these would block the sexual stage both in the human host and in the mosquito gut. Transmission blocking vaccines are not beneficial to the infected individual, but they might be found to be an essential part of malaria eradication programs.

Drawing from the previous information, it appears that the ideal vaccine should be multistage and multiantigen, able to stimulate high titres of antibodies against sporozoites, merozoites, and gametocytes, and cytotoxicity of T cells against infected hepatocytes.

1.1.5.2 Candidate antigens

The 23Mb *P. falciparum* genome (Gardner et al., 2002) encodes around 5300 genes and although various methods have been suggested to try to identify the most appropriate genes that should be included in a multigene vaccine (Doolan et al., 2003), no definite conclusion has yet been reached. Vaccine construction has so far relied on the evidence accumulated from studies of the immune responses present in naturally immune individuals, populations living in endemic areas and immune responses seen in mice and humans following injection of sporozoites.

Pre-erythrocytic stage antigens

Circumsporozoite protein (CS): This protein is the most abundant protein on the surface of sporozoite as well as in the intracellular hepatic stages of the parasite. Most vaccines have used CS as their target, mainly because naturally immune individuals have been found to have CTL against this protein (Sedegah et al., 1992).

Sporozoites surface protein 2 (SSP2) or Thrombospondin related adhesive protein (TRAP): Together with CS, SSP2 is also very abundant on the surface of the sporozoites. Malaria immune subjects have been found to have cytotoxic T cells against this protein (Aidoo et al., 1995) and similarly, volunteers immunised with irradiated sporozoites have been shown to produce a CTL response to it (Wizel et

al., 1995). Also, monoclonal antibodies to SSP2 were identified in mice immunised with *P. yoelii* (Rogers et al., 1992).

Liver stage antigen 1 and 3 (LSA1, LSA3): Both these proteins are expressed on the surface of infected hepatocytes. Naturally immune persons have been shown to have developed CTL responses to both of these antigens (Aidoo et al., 2000, Connelly et al., 1997, Doolan et al., 1997, Hill et al., 1992), and immunised volunteers are capable of developing the same type of response (Doolan et al., 1997).

P. yoelii hepatocyte erythrocyte protein (*PyHep17*): This protein is a homologue of the *P. falciparum* exported protein 1. PyHep17 has been used for numerous studies in mice where it was shown to be a target of protective antibodies and T cells, and immunisation with DNA expressing this protein can induce CD8 mediated (Doolan et al., 1996a) although, as for TRAP, no specific epitope has so far been identified.

Erythrocytic stage antigens

Merozoite surface protein 1(MSP1): MSP1 the best characterised antigen involved in erythrocyte invasion. Expressed in all species of plasmodium, it is responsible for parasite entry into cells. Antibody against this protein have been associated with resistance to clinical malaria (Egan et al., 1996), and can inhibit erythrocyte invasion (Weiss et al., 1998) as well as preventing parasite growth in vitro (Egan et al., 1999).

Apical membrane antigen 1 (AMA1): AMA1 is present in all species of plasmodium, is initially found in the apical organelles but then expressed on the surface of the merozoite where is involved in cell invasion. Its sequence has been conserved during evolution. Antibodies against this protein have been found in populations exposed to malaria (Thomas et al., 1994), and these antibodies have been shown to inhibit merozoite invasion *in vitro* (Hodder et al., 2001).

P. falciparum erythrocyte membrane protein 1 (*PfEMP1*): PfEMP1 allows adherence to endothelial cells but the problem is the very high level of polymorphism of this protein (Richie & Saul, 2002). PfEMP1 has been speculated to be a target for antibody mediated immunity since sera from immune individuals have high titres of antibodies against PfEMP1 (Miller et al., 2002).

Sexual stage antigens

Plasmodium antigen 25 and 28 (Pfs25, Pfs28): Pfs25 and Pfs28 are two transmission blocking antigens, present on the surface of the zygote and mature ookinete and found in many plasmodium species (Duffy & Kaslow, 1997, Tachibana et al., 2001, Tsuboi et al., 1998). They play important roles in ookinete survival in the midgut, penetration of the epithelium and transformation of the ookinete into the oocyst (Tomas et al., 2001). Antibodies against Pfs25 and Pfs28 can inhibit maturation of zygotes into ookinetes and of ookinetes into oocysts (Tsuboi et al., 1997) as well as blocking ookinete penetration of the mosquito midgut (Kaslow et al., 1994).

Using multiple antigens should allow for an additive effect of the immune response induced by each antigen independently, resulting in substantial immunity. Also, mixtures of antigens should allow immunity in genetically heterologous populations, and protect against more than one species of malaria, reducing the emergence of parasites that escape vaccine-induced immunity. Nonetheless, it is possible that using a mixture of antigens could cause interference between them, or even increase the possibility of local reactogenicity as the number of antigens in the vaccine increases.

The problems that have obstructed the development of a malaria vaccine so far include the polymorphism and the antigenic diversity in particular in blood stage antigens, although there are conserved regions within these. Also, the presence of cryptic epitopes within antigens has been problematical as well as the lack of appropriate *in vitro* correlates of protection, ie *in vitro* results have not always correlated with *in vivo* results and vice-versa. Finally, the lack of suitable animal models for testing the efficacy of vaccine candidate antigens is an extra complication, as well as the lack of correlation between stimulation of immune responses and protection (Chauhan & Bhardwaj, 2003).

1.1.6 Immunotherapy of malaria

The two stages of malaria infections that have mainly been targeted for vaccine design so far are the sporozoite and the liver stage.

1.1.6.1 Attenuated organisms

The first successful attempt to induce protective immunity was achieved by immunisation with irradiated sporozoites (Hoffman et al., 2002) (Herrington et al., 1991). This immunisation induces both cellular (Doolan et al., 1997, Wizen et al., 1995) and humoral (Nardin et al., 1982, Zavala et al., 1985) immune responses targeted at the sporozoite surface proteins, and has been shown to confer protection in over 90% of volunteers (Hoffman et al., 2002) in a T lymphocyte IFN γ dependent way (Hoffman et al., 1989). Although efficient, this is hardly a practical method, but the fact that the irradiated sporozoite vaccine was efficient indicated that it is advisable to use multiple antigens from the same stage of the disease, and has directed a lot of efforts to trying to develop a vaccine targeted at sporozoite stage immunity.

1.1.6.2 DNA vaccines

DNA vaccines consist of plasmids encoding the sequence of a protein or of multiple epitopes from a single or various proteins. These plasmids are then injected, often mixed with an adjuvant to enhance immunogenicity, and taken up by the local dendritic cells or muscle cells, where the protein is translated and peptides are presented.

DNA vaccines expressing only one protein, CS, can induce strong CD8⁺ IFN γ dependent and antibody responses in mice (Sedegah et al., 1994) and humans (Wang et al., 1998b) and confer partial protection that can be further improved by coinjection of a plasmid expressing GMCSF (Sedegah et al., 2000). Studies have subsequently reported similar results with a multi-antigen immunisation using two plasmids expressing the PyCS and PyHep17 both from the pre-erythrocytic stage (Doolan et al., 1996b), and then looking at a vaccine composed of 4 plasmids expressing proteins from the sporozoite (PfCS, PfSSP2) and from the liver stage (PfExp1, PflSA1). The four antigen vaccine was found to stimulate CD8⁺ CTL

responses to all proteins in both monkeys (Wang et al., 1998a) and humans (Doolan & Hoffman, 2001), encouraging the use of multi-antigen multi-stage vaccines, and proving that no competition is present between the different proteins. Finally, a DNA multistage-multigene vaccine programme is now aiming at investigating a mixture consisting of 5 plasmids encoding proteins from the sporozoite stage and 4 from the blood stage (Webster & Hill, 2003).

The efficiency of DNA immunisation has proved to only be partially protective, even upon expression of multiple antigens and from multiple stages, or even using additional plasmids expressing a GMCSF (Moorthy et al., 2004a). Moreover, DNA subunit vaccines require potent adjuvants that may result in adverse reactions in humans, suggesting that different methods of immunisation may be more efficient.

1.1.6.3 Peptide vaccines

Peptide vaccination mainly induces strong antibody responses and rather low cellular immunity in humans and animals, probably because the peptide is loaded directly onto the MHC molecule without undergoing the class I processing pathway. When co-immunised with a universal T helper cell epitope, or even as a long synthetic peptide in an oil based adjuvant, peptides are able to elicit specific CD4 and CD8 T cell responses (Lopez et al., 2001, Nardin et al., 2001, Nardin et al., 2000) and protect mice from sporozoite challenge in *P. yoelii* and *P. berghei* models.

Peptide vaccines based on a single antigen (CS or SSP2) conjugated to tetanus toxoid initially gave encouraging results in mice, and rabbits, though only resulted in partial protection in phase I and II clinical trials (Etlinger et al., 1988, Herrington et al., 1987, Nussenzweig & Nussenzweig, 1989, Sharma et al., 1996, Zavala et al., 1985). Following this, a vaccine containing multiple (T and B cell) epitopes from the CS repeat region was shown to elicit strong antibody responses both in mice, monkeys (Collins et al., 1997, Moreno et al., 1999) and humans (Lopez et al., 2001, Nardin et al., 2001, Nardin et al., 2000), but, similarly to DNA vaccines, single peptide antigen vaccines have never resulted in high levels of protection. This has focussed research on subunit vaccines using multiple

antigen peptides (MAP). The Spf66 vaccine was the first construct expressing sequences from three erythrocytic stage antigens (Moreno & Patarroyo, 1989, Patarroyo et al., 1987), but this vaccine still failed to provide consistent protection. Poor performance of this vaccine in Africa in phase III clinical trials showing similar cases of symptomatic falciparum malaria or any index of malaria in immunised and control groups in follow up trials shifted the attention to other alternatives (Acosta et al., 1999, Alonso et al., 1994, D'Alessandro et al., 1995, Maitland et al., 2003, Nosten et al., 1996, Patarroyo et al., 1992).

1.1.6.4 Recombinant proteins

Recombinant protein vaccines consist of injection of protein expressed in another organism. As for DNA or peptide vaccines, often it is not the whole protein that is expressed but only the immunodominant region. The advantage of this technique is that the protein can easily be fused to another protein that is known to induce strong responses (often Hepatitis B virus core protein), and can thus act as an adjuvant.

Recombinant protein vaccines for malaria initially used a single MSP1 purified (Perrin et al., 1984) or recombinant (Herrera et al., 1990) fusion protein showing partial protection in monkeys and humans (Keitel et al., 1999). Then, the immunodominant domain was expressed resulting in protective immunity in monkeys when specific adjuvants were used (Kumar et al., 2000). Similarly, apical membrane antigen 1 (AMA1) (Collins et al., 1994) and serine repeat antigen (SERA) (Inselburg et al., 1993, Inselburg et al., 1991), both from the merozoite stage, could only partially protect monkeys from parasite challenge. Not even a mixture of blood stage antigens (MSP1, MSP2, RESA) could confer protection upon blood stage challenge in volunteers (Lawrence et al., 2000), though a reduction in parasite density could be seen in previously exposed individuals (Genton et al., 2002). Vaccines targeting the sexual stage have also shown immunogenicity in mice and rabbits injected with a fusion protein of Pfs25 and Pfs28 (TBV25-28) (Gozar et al., 2001).

To date, the most successful vaccine has been the RTS,S/AS02. This is a recombinant protein formed by the fusion of hepatitis B surface antigen with most

the of CS antigen expressed in yeast where this fusion protein (RTS) binds the hepatitis B surface antigen (S). Two-three repetitive injections of this recombinant fusion protein mixed with the adjuvant AS02 have been shown to induce antibodies to the sporozoite stage. Unfortunately, although this vaccine can induce protection in 30-60% of volunteers in America upon sporozoite challenge (Kester et al., 2001, Stoute et al., 1997), and is not strain specific (Allouche et al., 2003), a clinical trial carried out in the Gambia (Bojang et al., 2001) has proved the response to be short lived (Bojang et al., 2001).

Other recombinant protein vaccines are currently undergoing clinical trials. ICC-1132 is the latest recombinant vaccine, an improvement of the RTS/AS02, consisting of T and B-cell epitopes from the repeat region of CS protein and a universal T cell epitope from the C terminus of the protein, expressed in the highly immunogenic recombinant hepatitis B virus core protein. This, prepared in adjuvants that would be suitable for humans, has so far been successful in mice and monkeys showing T cells and antibody responses, and is currently in phase I clinical trials (Birkett et al., 2002). Also, a construct expressing epitopes from nine *P. falciparum* stage specific antigens (sporozoite, liver, erythrocyte, and sexual stage) and a universal T epitope from tetanus toxoid expressed in baculovirus and purified, is currently being studied in non-human primates (Shi et al., 2000, Shi et al., 1999).

1.1.6.5 Viral vectors

Using a viral vector has the advantage of not requiring adjuvants and allowing expression of the recombinant protein inside the infected cells, therefore providing an efficient delivery to MHCI and MHCII antigen-processing pathways via endogenous pathways

Immunisation of mice with attenuated adenovirus expressing PyCS has resulted in IFN γ mediated protection response and decreased level of parasitaemia in the liver (Rodrigues et al., 2000, Rodrigues et al., 1998). An influenza virus vector expressing a CS epitope has also been tested but this was only shown to induce strong CTL responses (Li et al., 1993).

The most extensively used virus in malaria research, is vaccinia virus. Modified vaccinia Ankara (MVA) (Gilbert et al., 1999), and the highly attenuated NYVAC strain expressing the full length *P. berghei* CS protein can induce both antibody and cellular responses and confer CD8⁺T dependent protection against sporozoite challenge (Lanar et al., 1996). However, such recombinant viruses have mainly been used in prime-boost regimes (see later).

The most successful viral malaria vaccine so far has been the multigene, multistage NYVAC-Pf7 vector. This virus expresses seven full length or nearly full length *P. falciparum* proteins from the different stages of infection, in particular: three from the pre-erythrocytic stage (CS, TRAP, LSA1), three from the erythrocytic stage (MSP1, AMA1, SERA) and one sexual stage antigen of (Tine et al., 1996). NYVAC-Pf7 can successfully induce both antibody and CTL responses in monkeys (Tine et al., 1996) and in humans (Ockenhouse et al., 1998), resulting in one volunteer being completely protected and others showing clear signs of delayed infection upon sporozoite challenge.

1.1.6.6 Other vectors

CS has also been expressed as a full length gene in recombinant *Salmonella typhimurium*, successfully inducing protein specific CD8⁺ CTL protection against sporozoite challenge (Aggarwal et al., 1990, Sadoff et al., 1988). Moreover, TyVLP, consisting of a single protein species that can be produced in yeast, could induce protective CTL responses when expressing a string of 15 epitopes (Gilbert et al., 1997), although could not protect against challenge when expressing CS alone (Gilbert et al., 1999).

1.1.6.7 Prime-boost regimes

Single immunisation or repeated homologous administration of DNA, or recombinant virus does not produce the levels of CD8⁺T cells required for protection against malaria in murine models (Gilbert et al., 1999, Lanar et al., 1996, Prieur et al., 2004, Pye et al., 1991, Schneider et al., 1998, Sedegah et al., 1990). The overall immunogenicity of a virus is greater than a plasmid or a protein, however, when used alone or in homologous prime-boost regimens, much

of the immunogenicity of these recombinant viruses is targeted at the vector components. Heterologous prime-boost vaccination, using different vectors to prime and to boost has therefore been identified as the best strategy to induce immune responses.

DNA, viral vectors, as well as TyVLP (Allsopp et al., 1996, Gilbert et al., 1999) have been tested for their ability to prime and to boost an immune response, but the sequence with which they are used determines the efficiency of the boost (Li et al., 1993, Sedegah et al., 1998). Viral vectors such as fowlpox 9 (FP9) can usually be used both for priming or boosting (Anderson et al., 2004, Moorthy & Hill, 2002). However, MVA is not efficient at priming (Li et al., 1993). DNA has been shown to be efficient only if given first (Gilbert et al., 1999, Schneider et al., 1999).

DNA vaccination is a very effective priming tool, and a DNA priming-MVA boosting regime has proved very efficient, leading in some cases to complete protection (Schneider et al., 1998). In addition, non replicating adenoviruses (Bruna-Romero et al., 2001), or recombinant influenza virus expressing PyCS have also been tested as priming vectors, and when followed by a dose of a highly attenuated vaccinia virus expressing the same epitope or gene, were shown to induces a high degree of vaccine protection (Li et al., 1993, Rodrigues et al., 1994).

DNA, recombinant MVA or FP9 have been constructed expressing ME-TRAP. This is a multiple epitope (ME) construct consisting of a string of B and T cell epitopes from the pre-erythrocytic antigens fused to the whole SSP2 (TRAP) antigen. Various prime-boosting regimes have been tried using DNA (Moorthy et al., 2003a) or FP9 (Moorthy et al., 2003b) as priming vectors showing safety in humans and the ability to stimulate immune responses in animals. This vaccination gave promising results, although vaccination with the RTS,S/AS02 still seems to be more protective probably due to the presence of antibody responses as well as cellular responses (Kester et al., 2001). Current studies are therefore assessing the effect of using MVA expressing CS in a prime boost regime with the peptide RTS/AS02 vaccine.

MVA has also been tested recently, together with DNA and FP9, as a boosting factor for another polyantigen vaccine (L3SEPTL) formed from six preerythrocytic antigens expressed either by DNA, MVA, or FP9 (Prieur et al., 2004).

Similarly to MVA, NYVAC is also capable of boosting the immune response, and protection against CS in mice (Sedegah et al., 1998), and in rhesus macaque immunised with a vaccine expressing two pre-erythrocytic antigens and two blood stage antigens (Rogers et al., 2001). Finally, similarly to the other approaches, the effectiveness of boosting can be further improved by including expression of immunostimulatory cytokines as GMCSF (Sedegah et al., 2000).

Although there is still no vaccine against malaria, all these studies have helped in reaching the conclusion that the ideal vaccine should be able to stimulate a CTL response as well as antibody responses, and include multiple epitopes from one protein as well as multiple antigens. A multistage, multiantigen vaccine using full-length genes and delivered as heterologous prime boost regime including a viral dose appears to be ideal. Antigens to be included would be CS, TRAP, LSA1, MSP1, RESA, SERA, PfEMP1, Pf25 and Pf28 together with an immunostimulatory cytokine.

1.2. Dendritic cells

All cells in the body are antigen presenting cells (APC) since they can present peptides from internal antigens on MHCI molecules. Dendritic cells (DCs), together with macrophages, are specialised antigen presenting cells in that they also express MHCII molecules on which they present peptides from antigens taken up from the environment. Additionally, dendritic cells are considered the best antigen presenting cells in that they are the only ones able to stimulate naïve T cells (Steinman, 1991).

1.2.1 Dendritic cell development

Dendritic cells originate in the bone marrow and are found across the body in an immature form where they act as sentinels, monitoring the environment, waiting to encounter a foreign antigen that they can process and present to cells of the immune system.

Dendritic cells are scattered around the body and differ in phenotype and activity depending on their location. Two main lineages of DCs have been identified in both humans and in mice, these are myeloid, and lymphoid cells. A third group, the plasmacytoid DCs, has also been characterised but whether this represents a different lineage or derives from the lymphoid lineage is still not clear. Both lymphoid and myeloid cells originate from a haemopoietic stem cell in the bone marrow that can give rise to a common myeloid precursor (CMP), or a common lymphoid precursor (CLP) depending on the cytokine available. Granulocyte-monocyte-colony-stimulating-factor (GM-CSF) is the main inducer for the development of myeloid cells whereas TNF α or IL7 seem to be the cytokines triggering lymphoid cell differentiation (Manz et al., 2001). These cytokines can also be used in vitro to obtain the desired lineage.

The immature precursors leave the bone marrow, enter the blood stream and migrate to sites of potential antigen entry where, according to the cytokines present in the tissue, they differentiate and become immature resident tissue cells. Despars et al (Despars & O'Neill, 2004) have described this process with a “niche” model relying on the presence of different cytokines. Each organ would

contain various niches and different precursors within which the different DCs can develop. For instance, the CMP gives rise to Langerhans cells in the skin under the influence of TGF β , but will also develop into interstitial cells in the epidermis. Residing in an immature state, these DCs patrol for the possible presence of antigens. Lymphoid cells arise from the CLP in the presence of IL3 whereas Flt3 seems to preferentially induce production of plasmacytoid DCs (Figure 1.2).

Cells derived from myeloid precursors develop into Langerhans, dermal, and interstitial cells, all present in peripheral tissue. Those derived from lymphoid precursors give rise mainly to cells that are present in primary and secondary lymphoid organs, often responsible for T cell negative selection or involved in downregulation of primary peripheral T cell responses. Plasmacytoid DC are found in spleen (Grouard et al., 1997), bone marrow, blood (Liu et al., 2001b) and thymus (Bendriss-Vermare et al., 2001) and seem to originate from both myeloid and lymphoid precursors (Ardavin, 2003). Follicular DC, despite their name, are not related to those that originate from the BM, they are present in the LN and retain immune complexes over a period of months in their native conformation and are important for memory.

In mice six DC populations have been defined (Shortman & Liu, 2002), all expressing CD11c on their surface but differing for other surface markers (Figure 1.2). Myeloid cells can be distinguished from lymphoid cells in that only myeloid cells express CD11b whereas the presence of CD8 α on the surface denotes cells of the lymphoid lineage. The spleen contains both myeloid (CD8 α ⁻) and lymphoid cells (CD8 α ⁺) but whereas lymphoid cells are all negative for CD4, myeloid cells are further divided into CD4⁺ and CD4⁻ cells. In addition to these three cell types found in the spleen, two more are present in the LN. One type of cells corresponds to migrated Langerhans cells, therefore myeloid, expressing CD11c, CD11b, not CD8 α nor CD4, but with additional expression of langerin and CD205. The second extra population found in the LN is believed to be the mature form of interstitial DCs, thus again myeloid cells CD11c⁺CD11b⁺CD8 α ⁻CD4⁻, but expressing moderate levels of CD205 on their surface. Finally the sixth

type of DC present in mice is the plasmacytoid cells characterised by the B220 marker and negative for CD11b while expressing CD8 α (CD11c⁺CD11b⁻CD4⁻CD8 α ⁺B220⁺)

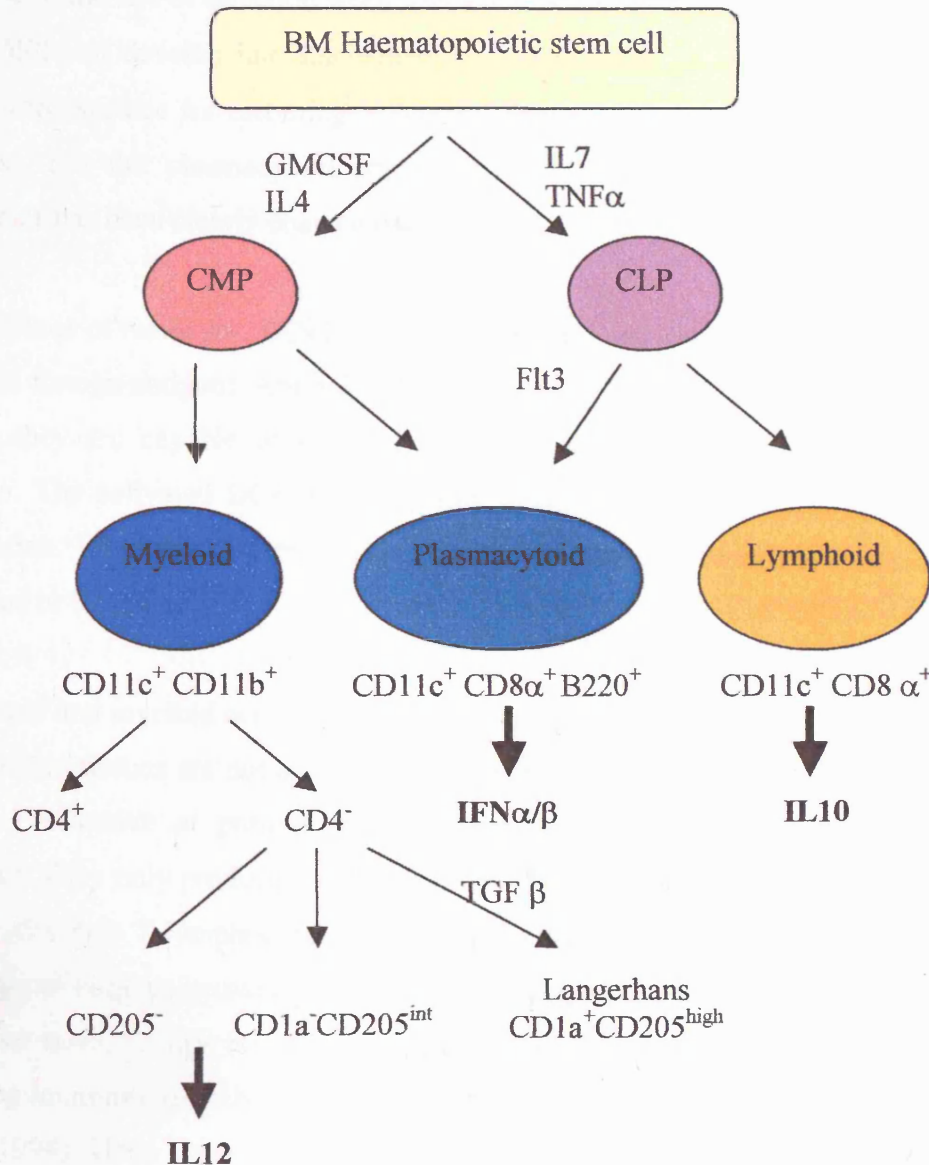


Figure 1.2: DC subsets development in mice

Myeloid, plasmacytoid and lymphoid cells derive from two precursors that develop from a bone marrow (BM) haematopoietic precursor: common myeloid precursor (CMP) or common lymphoid precursor (CLP) and further differentiate into cells expressing different surface markers and secreting different cytokines. All six types of cells are found in mice lymph nodes.

The types of human DCs have not been studied as extensively as those from mice, and the presence of a lymphoid lineage is still controversial. All human DCs are characterised by expression of CD34a. The myeloid precursor develops into monocytes that are found in the blood stream where they patrol the body and migrate to the site of infection when necessary. Alternatively, the CMP stimulated by TGF β can develop into Langerhans cells that reside in the skin where they inspect the surface for incoming pathogens. Lymphoid cells have not really been defined, but the plasmacytoid population probably arising from a lymphoid precursor has been clearly characterised.

In the tissue of residence, DCs have high phagocytic activity and can take up both self and foreign antigens. Antigen uptake induces the cell into an activated state in which they are capable of inducing tolerance or an immune response to the antigen. The activated DCs start travelling to the LN that they reach upon full maturation. There they present the antigen to T cells and either induce an immune response or tolerance.

Lymphoid and myeloid cells differ in their function. Lymphoid cells found mainly in lymphoid tissues are not as efficient as myeloid cells at phagocytosis although they are effective at presenting antigens taken up from dying cells (cross-priming). They only produce limited amounts of IL12 (needed for autostimulation and to stimulate T lymphocytes) and are rather inclined to induce tolerance and Th2 type of responses inducing IL10 release from CD8 cells. Myeloid cells, on the other hand, though not efficient at cross-priming, are the most efficient at inducing immunity to pathogens by reacting with, and activating T cells (Hannum et al., 1994). Upon activation myeloid cells release large amounts of IL12 and IFN γ , resulting in Th1 type immune response that activates CD8⁺T cells. Plasmacytoid cells are considered the natural interferon producing cells, releasing type I IFN.

Most research using DCs uses human monocyte cells derived from blood monocytes cultures including GM-CSF and IL4, or the corresponding myeloid

cells amplified from mouse bone marrow with GM-CSF since these are the cells which usually react to incoming pathogens.

1.2.2 Antigen uptake by dendritic cells

The immature DC found in the periphery at locations where antigens are likely to enter the body, are very efficient in capturing and processing antigens (Steinman & Swanson, 1995). Mature DCs, on the other hand, predominantly found in lymph nodes, where contact with other lymphocytes is possible, are poor at taking up antigens but very efficient at interacting with cells and initiating an immune response (Lanzavecchia & Sallusto, 2000). Antigen uptake triggers cell maturation resulting in immature DCs losing the receptors required for the capture of antigen (Fc γ and Fc δ receptors) in favour of migration and T cell stimulatory functions (figure 1.3) (Lukas et al., 1996).

DCs continuously sample the antigenic content of their environment and can capture antigens via three different mechanisms. Particles and microbes can be phagocytosed (Banchereau & Steinman, 1998) which appears to be particularly efficient (Inaba et al., 1998). Alternatively, DCs can take up extracellular fluids and solutes by macropinocytosis, by the formation of a pinocytic vesicle (Sallusto et al., 1995, Steinman & Swanson, 1995). Finally, DCs can use adsorptive endocytosis that involves using C-type lectin receptors such as the macrophage mannose receptor (Sallusto et al., 1995) and DEC-205 (Jiang et al., 1995) as well as Fc γ and Fc ϵ receptors (Albert et al., 1998, Sallusto & Lanzavecchia, 1994) present on the cell surface. Although endocytosis appears to be downregulated after antigen uptake while the cell undergoes maturation, clathrin mediated uptake has been shown to still be possible even in mature cells (Mellman & Steinman, 2001).

When they encounter local inflammatory mediators, such as TNF α or LPS, or when they take up an antigen, DCs become activated and undergo a series of physiological changes leading to terminal differentiation into potent professional APC (Banchereau & Steinman, 1998) coinciding with the ability to migrate to regional lymph nodes (Sallusto & Lanzavecchia, 1994).

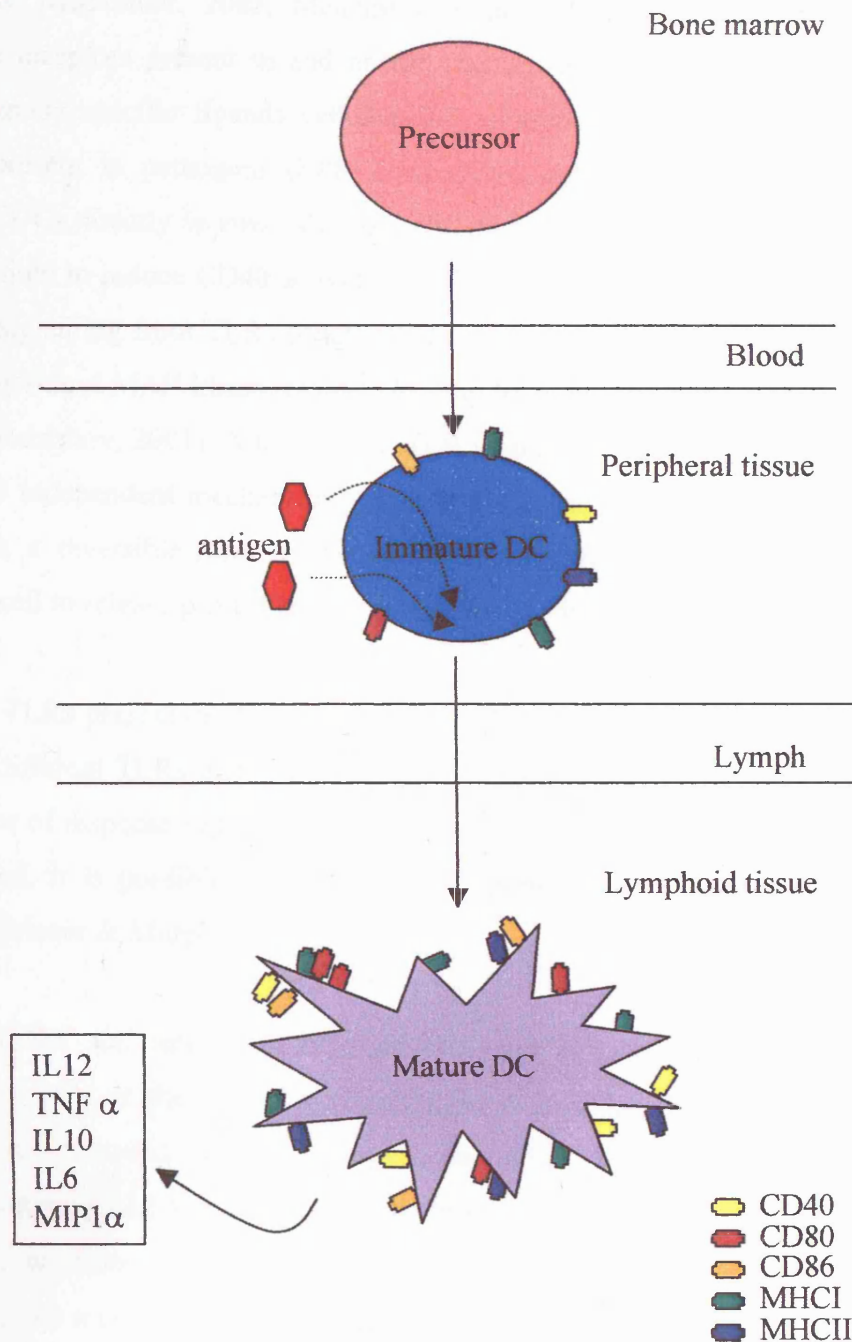


Figure 1.3: Life cycle of dendritic cell

The bone marrow precursor migrates to peripheral tissues where the immature DCs develop and upon encounter and uptake of antigen these mature upregulating surface markers and enter the lymphatics to reach the lymphoid tissues where the mature DC can interact and induce T cell activation via costimulatory molecules and released cytokines.

1.2.3 DC Activation

DCs recognise bacteria, viruses and parasites via Toll like receptors (TLRs) (Janeway & Medzhitov, 2002, Medzhitov et al., 1997), a family of pattern recognition receptors present in and on the surface of antigen presenting cells. TLRs recognise specific ligands called pathogen-associated molecular patterns (PAMPs) present in pathogens (LPS, CpG ODN, dsRNA). TLR ligands can activate the DCs directly *in-vivo*. Alternatively, the presence of activated CD4⁺Th cells is required to induce CD40 activation of DC (Toes et al., 1999); (Bonifaz et al., 2002). Signalling from TLRs usually takes place through the MyD88 adaptor protein triggering a MAP kinase cascade leading to gene transcription (Kawai et al., 1999, Medzhitov, 2001). Alternatively, TLR ligands can also activate the DCs in a MyD88 independent mechanism (Akira et al., 2001, Medzhitov, 2001). DC activation is a reversible state that does not involve phenotypic changes but induces the cell to release pro-inflammatory cytokines for T cell activation.

The type of TLRs present on the surface of the cells varies depending on the DC subset, and different TLRs differ in their ligands and response they induce (table 1.1). The type of response of the cell therefore depends on the stimulus and on the TLR activated, it is possible that different DC subsets determine Th1 or Th2 polarisation (Moser & Murphy, 2000).

TLR2 and TLR4 are present on myeloid DC, and can both bind LPS and purification of LPS or the use of synthetic lipid A revealed that TLR4 is the primary receptor involved in DC activation upon LPS stimulation (Hirschfeld et al., 2000). Binding of LPS to these receptors triggers IL12 release and minimal IL10 release, together with TNF α and IL6 (Thoma-Uszynski et al., 2000). Production of this set of cytokines polarises CD4 cells into a Th1 type response, this is how microbial pathogens activate cell-mediated Th1 immunity. On the other hand, plasmacytoid DC have TLR7 and TLR9 on their surface, the first binding to viruses and the latter to viral DNA. Binding to these receptors induces the cell to release IFN α and not IL12 therefore triggering an antiviral Th2 type response that does not involve IL12 (Jarrossay et al., 2001, Kadowaki et al., 2001, Krug et al., 2001).

Toll Like Receptor	Ligand	Reference
TLR2	Components of gram –ve bacteria Mycobacteria Yeast cell wall and gram +ve bacteria	(Jankovic et al., 2001) (Jankovic et al., 2001) (Aderem & Ulevitch, 2000)
TLR3	Double stranded DNA	(Steinman & Pope, 2002)
TLR4	LPS Gram-ve bacteria (except some components) Respiratory syncytial virus	(Jankovic et al., 2001) (Jankovic et al., 2001) (Jankovic et al., 2001)
TLR5	Bacterial flagellin from gram -ve and gram +ve bacteria	(Jankovic et al., 2001)
TLR6	Yeast cell wall and gram +ve bacteria	(Aderem & Ulevitch, 2000)
TLR7	Viruses	
TLR9	Unmethylated CpG containing DNA	(Jankovic et al., 2001)

Table 1.1: TLR specificity.

1.2.4 Antigen processing

Activation of DC by antigen uptake is followed by antigen presentation and cell maturation. Unlike activation, maturation is an irreversible state characterised by upregulation of antigen presenting molecules (MHCI and MHCII), and costimulatory markers (CD40, CD80, CD86), as well as by the secretion of cytokines from the DC (IL12, MIP1 α , IL1, IL6, IL10, TNF α) (Figure 1.3). These modifications allow for maximal T cell activation from the DC. Antigens are presented on the surface of DCs as peptides associated with MHCII or MHCI molecules, depending whether the antigen was taken up from the environment, or from the cytosol respectively.

1.2.4.1 MHCII antigen presentation:

MHCII are formed in the rough endoplasmic reticle where the newly synthesised MHCII molecule associates with an invariant chain in the peptide binding cleft (figure 1.4). This complex is transported through the Golgi and from the trans-Golgi the invariant chain targets the MHCII to the late endosomes where it meets the internalised antigen. The antigen degradation process takes place in endosomes through a process of increasing acidic pH. In the late endosome, both the invariant chain and the antigen are degraded, this allows for the antigenic peptides to replace the invariant chain in the cleft of the MHCII.

The rate of production of MHCII molecules does not change during cell maturation, instead, in immature cells the large numbers of MHCII produced are sequestered intracellularly into lysosomes (Cella et al., 1997). The invariant chain that binds in the groove of newly formed MHCII molecules also contains a cytoplasmic domain with a lysosomal targeting signal. Degradation of the lysosomal targeting signal is only possible in mature cells where the activity of an anti-protease (cystatic C) is reduced in favour of a protease (catS) activity. This results in higher amount of MHCII avoiding the lysosomes and instead reaching the membrane (Pierre & Mellman, 1998). The vesicle that reaches the membrane contains both the MHCII-peptide complexes and CD86 molecules that remain close to the complex upon exposure, thus conferring an ideal field for T cell

activation which remains stable for days (Cella et al., 1997, Mellman & Steinman, 2001, Pierre et al., 1997).

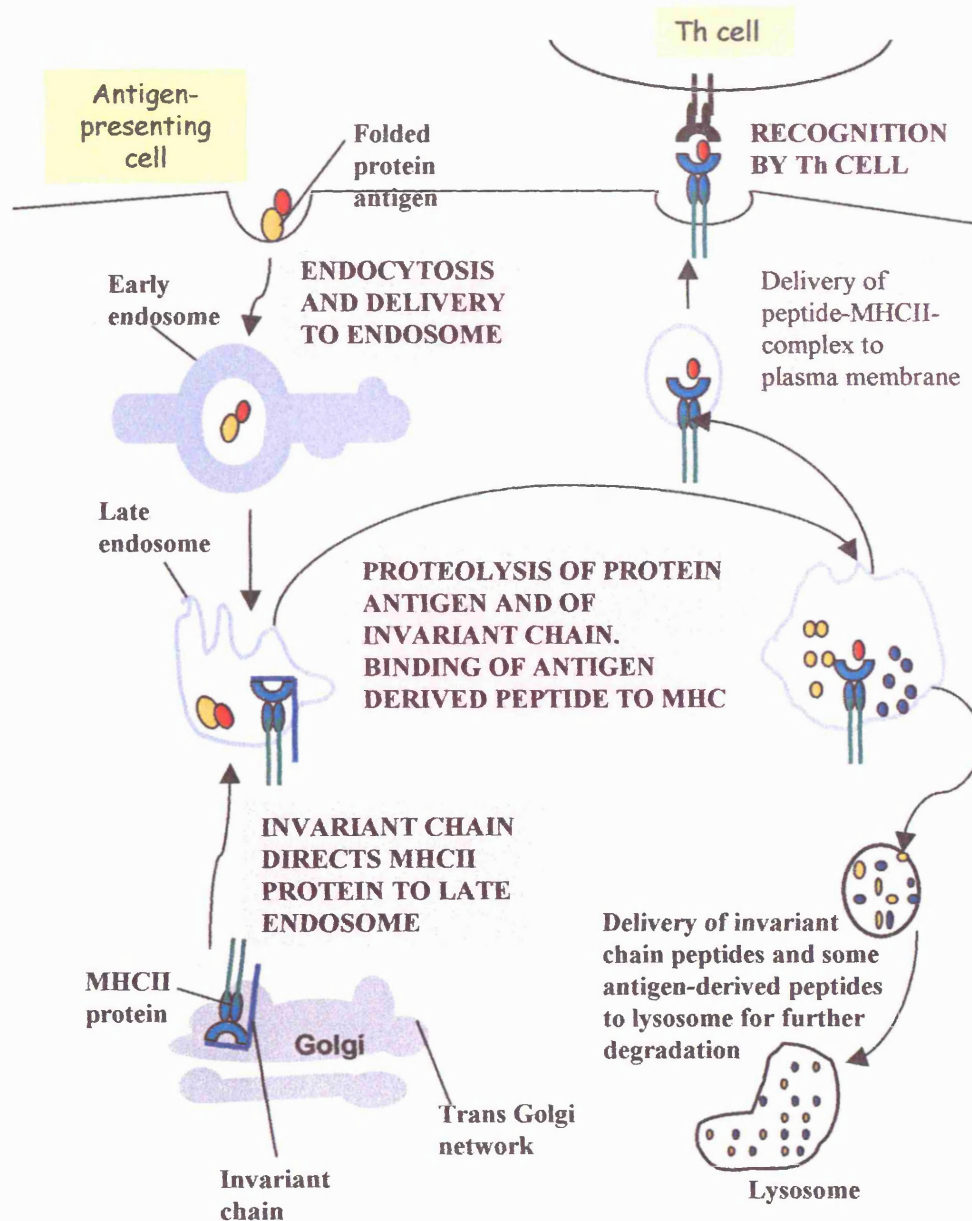


Figure 1.4: MHCII presentation pathway

Antigens are taken up from the environment and are internalised and processed into endosomes where they are then loaded onto MHCII molecules by replacing the invariant chain. The MHCII peptide is then delivered to the surface of the DC. MHCII molecules that are not loaded with antigen, as well as degraded invariant chain and peptides that are not presented are further degraded into lysosomes.

1.2.4.2 MHC I antigen presentation:

Presentation of peptides on MHCI is needed to activate CD8⁺ CTL cells. Loading on the MHCI molecules usually takes place for antigens synthesised within the cell including those derived from infecting viruses that express the proteins inside the cell, or even proteins that have been taken up upon phagocytosis of dying cells (cross-presentation) (Albert et al., 1998). The endogenously synthesised proteins are cleaved by proteasomes, and the peptides transported by means of transporters for antigen presentation (TAP) into the rough ER. Here, the peptides are bound to the newly formed MHCI molecules that then leave via the Golgi to the surface of the cell (Pamer & Cresswell, 1998) (figure 1.5).

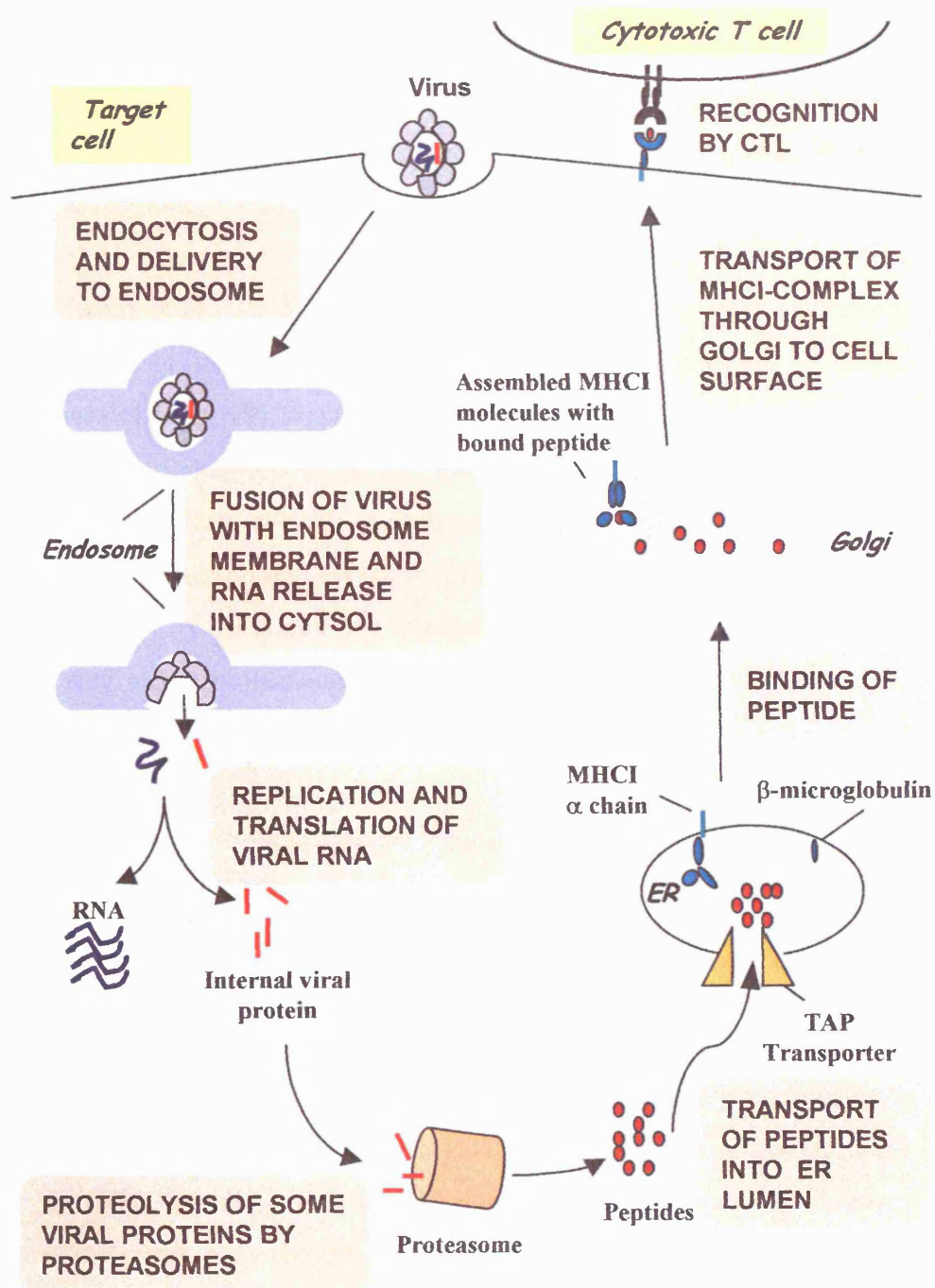


Figure 1.5: MHC I presentation pathway

Cytoplasmic antigens are processed through a proteasome and transported into the ER by means TAP transported. In the ER the peptides are loaded onto MHC I molecules and then through the Golgi for presentation on cell surface.

1.2.4.3 Cross presentation:

Cross presentation is the ability of DC to present in the context of MHCI, peptides from pathogen infected cells, and prime CTL responses against these intracellular microbial infections (Jung et al., 2002). Some endocytosed antigens derived from immune complexes, inactivated microbes, or antigens originally synthesised in other cells (cross-presentation) can gain access to the MHCI pathway (Bevan, 1976, Heath & Carbone, 2001). The uptake depends on selective endocytic uptake receptors on the surface of the DCs and presentation is usually TAP transporter dependent (Huang et al., 1996, Regnault et al., 1999) relying on a specialised phagosome-to-cytosol pathway in BMDC (Kovacsovics-Bankowski & Rock, 1995, Rodriguez et al., 1999). An endocytic vacuole is formed by FcγR that bind immune complexes and antibody coated tumour cells. Alternatively, the vacuole can form from the integrin and the phosphatidylserine receptor binding to dying cells, or even heat shock protein receptors binding their proteins. Antigens leave the vacuole probably by the action of a transporter and the ubiquitinated proteins are then targeted to the proteasome for cleavage and subsequent TAP mediated transport to then undergo the normal MHCI loading pathway.

Necrotic cells release their toxic content, peptides and proteins in the surrounding environment causing inflammation. Apoptotic cells, on the other hand, die keeping all the substances and organelles confined within them. Because of their ability to induce inflammation, it was initially thought that only necrotic cells could stimulate DC activation and cross-presentation (Gallucci et al., 1999, Sauter et al., 2000). The phenomenon of cross-presentation implies that a peptide initially presented on MHCI is finally presented on MHCII of a DC. Again, necrotic cells were thought to be the only ones to stimulate this process since the released peptides could be taken up by the DCs and thus presented via the MHCII pathway. Recently it has been shown that DCs recognise apoptotic cells and these are taken up, thus resulting in DC activation and presentation on MHCII of antigens initially present on the MHCI of the dying cell.

The MHCI and MHCII complexes interact with the CD4 and CD8 respectively on Th and CTL cells. This and other connections formed between DC and T cell

surface such as binding to TNF receptor family (eg. CD40) activate NF- κ B transcriptional factors, resulting in release of inflammatory cytokines, upregulation of surface molecules, and activation of CD4⁺T, CD8⁺T or B cells.

1.2.5 DC maturation and migration

In addition to antigen presentation, the process of DC maturation also involves changes in the panel of surface costimulatory molecules and receptors to induce the cell to travel to the lymph node for interaction with T cells. Maturation and migration occur concomitantly, to allow the DC to reach the LN in a fully mature state.

DCs migrate in response to chemotactic stimuli. The CC receptor molecules (CCR) on the cell surface induce the cell to migrate towards sites with a high concentration of their ligand. During maturation the DCs lose CCR5 and CCR2 that respond to inflammatory cytokines thus targeting the cell at the site of infection, in favour of expression of CCR7 that responds to chemokines expressed in the lymphatic vessel and lymphoid organs (Forster et al., 1999). Initially, the DC enters the lymphatics where the CCR7 ligand 6Ckine is produced. Subsequently, the cells are driven to the draining LN where MIP3 β (another CCR7 ligand) is expressed (Sallusto & Lanzavecchia, 2000).

Activation of T cells in the LN is also dependent on chemokines. The fully mature DCs that reach the LN attract T cells by releasing chemokines. Thymus and activation regulated chemokine (TARC) (Lieberman & Forster, 1999) and macrophage derived chemokine (MDC) (Godiska et al., 1997) are both produced by the DCs, bind CCR4 and CCR7 expressed by naïve T cells, and are involved in attracting memory cells (Sallusto et al., 1999). DC-CK1 is also a CC chemokine produced in secondary lymphoid organs by DC. This chemoattracts CD4 and CD8 T cells and is involved in priming naïve cells (Adema et al., 1997).

This ability of the DCs to travel is also exploited by viruses as a means of transport around the body to LN (Masurier et al., 1998).

1.2.6 Dendritic cell stimulation of the immune system

1.2.6.1 Activation of T cells

Dendritic cells have the unique ability to prime naïve T cells (Banchereau & Steinman, 1998). Mature DCs interact with ligands on the T cell (table 1.2). Following these interactions, both DCs and T cells release cytokines to maintain their activation state, and, depending on the type of pathogen that activated the DC (§ 1.2.1.3), these released cytokines polarise CD4⁺T cells into either Th1 or Th2 (d'Ostiani et al., 2000). Viruses, intracellular pathogens and bacteria induce DCs to release IL12 that stimulates Th1 differentiation whereas multicellular parasites stimulate Th2 differentiation.

Dendritic cell	CD4 ⁺ T cell
CD40	CD40 L
CD80	CD28
CD86	CD28
ICAM 1	LFA1
DC-SIGN	ICAM 3
LFA3	CD2
MHCII	CD4/ T cell receptor

Table 1.2: Interactions that take place between a mature DC and a naïve CD4⁺ cell

The CD40-CD40L interaction is the strongest stimulus. This prompts further upregulation of CD80 and CD86 on the DC, and induces release of cytokines some of which (eg. TNF and IL1) keep the DC in an active state (Cella et al., 1996, Miga et al., 2001), and others (IL1, IL6, MIP1 α , IL12) that activate CD4⁺T cells, B cells or CD8⁺T cells. Most importantly, the CD40-CD40L interaction stimulates DCs to secrete high levels of IL12. IL12 is the mediator between innate (NK cells) and adaptive (T and B cells) immunity (Trinchieri, 1998). CD4Th cells that recognise MHCII and IL12 are activated into IFN γ producing Th1 cells (Koch et al., 1996). Mice that lack IL12, IL12 β 1 receptor or the signal transducer and activator 4 (STAT4) have impaired IFN γ release (Trinchieri, 1998). IL12 also directly activates NK cells to secrete IFN γ . The IFN γ released by Th1 cells and NK cells in turn stimulates DCs to produce more IL12 (Snijders et al., 1998).

Together IFN γ and IL12 therefore maintain activation of the T cells and DC, and in some cases this stimulus has been shown to be enough to maintain T cell activation in an inversely proportional way to the presence of costimulatory molecules interaction (Jankovic et al., 2001). The importance of IL12 production can be seen from the fact that maintenance of T cell response in infection is due to constant presence of this cytokine (Yap et al., 2000), and that its receptor is expressed throughout the whole Th1 cell life cycle (Trinchieri, 1998).

The IFN γ produced by the polarised Th1 cells increases the responsiveness of T cells to IL12 (Smeltz et al., 2002) and the number of naïve cells developing into IFN γ producers (Creusot et al., 2003). IFN γ activates the anti-microbial activity of macrophages and together with IL12 promotes activation of CD8⁺CTLs. Th1 cells also release IL2 that induces CD8⁺T cell to secrete IFN γ that stimulates Th1 differentiation by positive feedback (Mailliard et al., 2002) (figure 1.6). Additional cytokines which induce and maintain Th1 responses include IL18, IL23 and type I interferons (Aliberti et al., 2000, Jankovic et al., 2001).

CD8 can also be activated by type I IFN independently of IL12 and, in the absence of IL12, these chemokines can, in human, directly polarise Th1 responses (Jankovic et al., 2001). Overall, DCs usually activate CD8⁺T cells via IL12 activation of CD4⁺Th1 cells that secrete IFN γ to act on the CD8⁺T cells (three-cells-system), but cytokines released by the activated DC (eg. IL12) may act directly on CD8⁺T cells inducing their activation.

Polarisation of CD4⁺T cells into Th2 responses are particularly dependent on CD28 binding to CD86, this in fact stimulates STAT6 and GATA3 genes expression (Oki et al., 2000) leading to IL4, IL5, IL13 expression (Jankovic et al., 2001) while downregulating IL12R. The main cytokine associated with Th2 responses is IL4, but, although IL4 is needed, Th2 polarisation can happen also when IL4R and STAT6 are not involved (Jankovic et al., 2000). Th2 cells secrete IL4, IL5, IL9, IL10 and IL13, expressed in different combinations depending on the infecting pathogen (Jankovic et al., 2001) (Figure 1.6). Their main role is to stimulate growth and differentiation of mast cells and eosinophils and also to

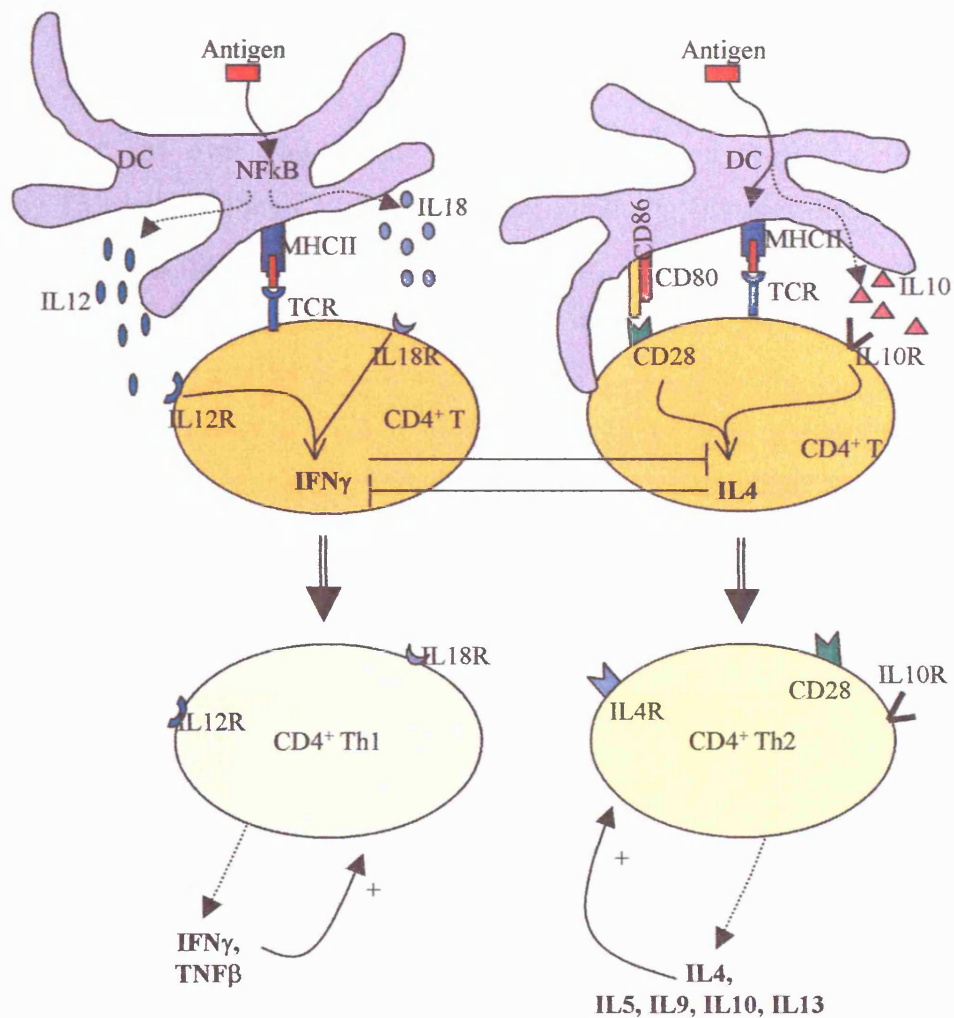


Figure 1.6 CD4 Th polarisation:

Depending on the stimulus received by the DC, this is stimulated to release IL12 and IL18 therefore polarising naïve CD4⁺T cells into Th1 cells that release IFN γ . Alternatively, DCs are stimulated to release IL10 and by interaction with CD28 stimulate the naïve CD4 to release IL4 that polarises the cell into Th2.

induce production of IgE that mediate activation of these cells (Jankovic et al., 2001) while inhibiting Th1 polarisation by producing IL10. Cytotoxic T lymphocyte Ag 4 (CTLA4) is a ligand that acounterbalances Th2 responses, this in fact inhibits activation of the cells by binding to CD80 and CD86 (Jankovic et al., 2001).

Maintenance of Th1 over Th2 activity is a balance between cytokines. IFN γ secreted by Th1 cells inhibits Th2 differentiation. Similarly, IL4 acts directly on Th1 cells to inhibit their differentiation. Moreover, IFN γ inhibits DC from secreting IL10 that has an inhibitory effect on IL12, whereas IL4 and IL10 inhibit IL12 secretion (Koch et al., 1996, Ria et al., 1998).

Nonetheless, the role of cytokines is not clear cut. IL12 also stimulates B cells to produce IL6 and IL10 that are required for Th2 differentiation (Skok et al., 1999). Similarly, IL4 has also been shown to be able to promote release of bioactive IL12 from human and murine DC (Jankovic et al., 2001), and may act in conjunction with IL12 to induce Th1 responses (Biedermann et al., 2001).

1.2.6.2 Activation of B cells

DCs can induce B cell proliferation either directly (Dubois et al., 1997) or indirectly via stimulation of Th cell.

Indirect activation of B cells:

Induction of a B cell response via T cell activation requires interaction of both B and T cells on the same DC and production of IL6 by the DC. B cells express CD40 on their surface and this can interact with the CD40L on the surface of T cells. The IFN γ released by Th1 cells can then induce B cells to produce IgG2 antibodies to mediate antibody dependent cellular cytotoxicity (ADCC). Th2, on the other hand, release IL4, IL5 and IL13 that induce B cells to produce IgE antibodies. These bind to antigen and to Fc receptors on the membranes of basophils, eosinophils and mast cells stimulating their degranulation that results in killing of the invading organism (Barton & Medzhitov, 2002) (Figure 1.7).

Direct activation of B cells:

Alternatively, DCs have been shown to be able to directly activate naïve and memory B cells (Banchereau et al., 2000, Dubois et al., 1998, Kikuchi et al., 2000). Naïve B cells previously stimulated by CD40L can respond to soluble factors secreted by interstitial DC, mainly IL12 (Dubois et al., 1998, Dubois et al., 1997). IL10 and TGF β for example can induce IgA secretion (Fayette et al., 1997). In addition, DC can enhance differentiation of CD40 activated memory B cells by stimulating the IL6 dependent activation pathway of B cells. DCs can produce soluble IL6R (also called sgp80) that binds the IL6 released by the B cell forming a complex with high affinity to the constitutive transducing chain (Dubois et al., 1998).

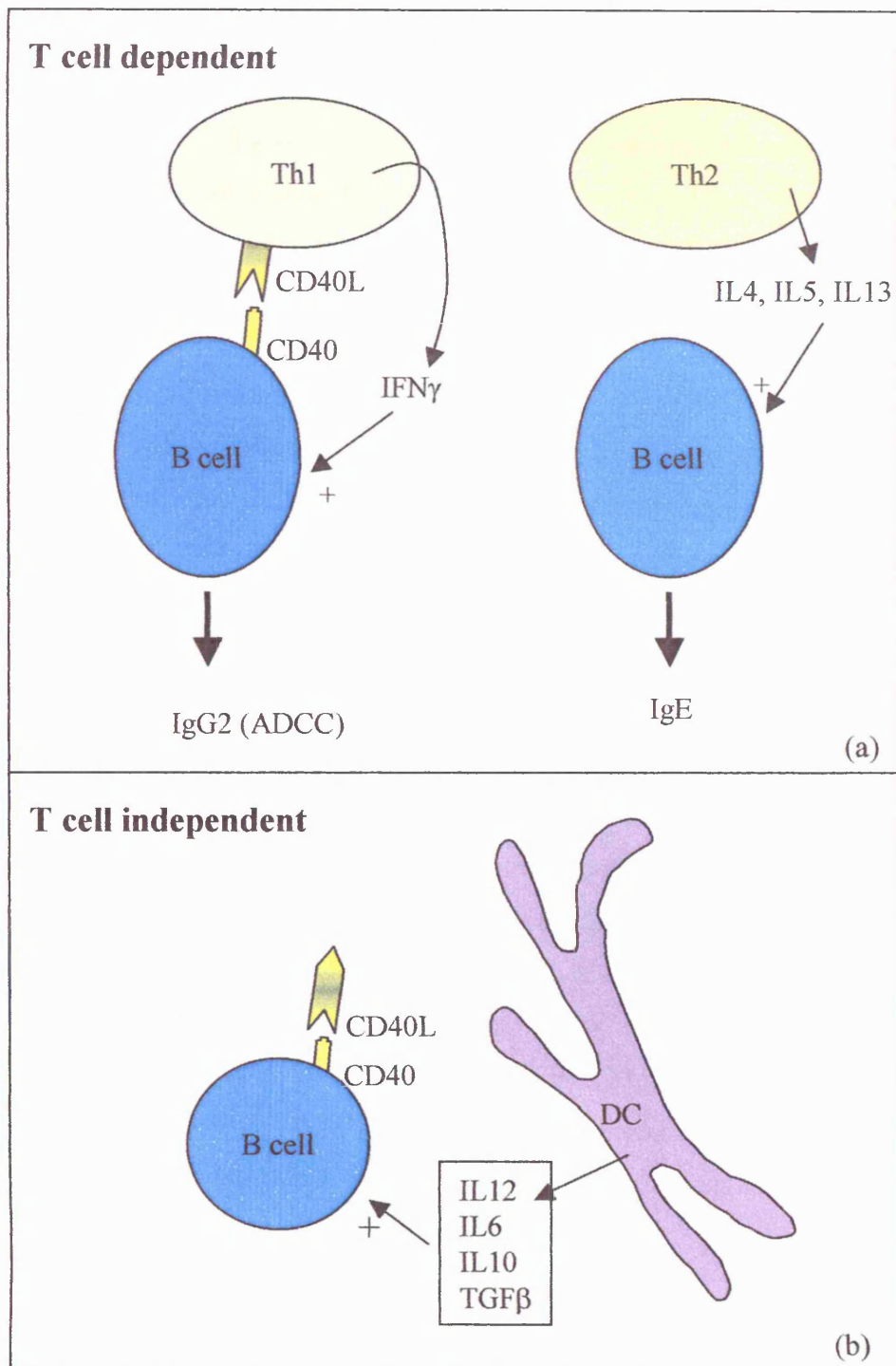


Figure 1.7 B cell activation

(a) B cell activation in Th dependent way involving different cytokines depending on the Th subtype

(b) Ability of DCs to activate directly B cells without requiring help by T cells

1.2.6.3 Activation of complement system

Immature DCs can activate the complement pathway by producing the C1q element that can bind apoptotic cells and trigger the complement cascade, but its production is downregulated upon DC maturation.

1.2.6.4 Induction of tolerance

In addition to pathogenic antigens, DCs may also take up and present self-antigens where they are required not to activate immune responses, but to silence them. Although T cell tolerisation usually takes place in the thymus where T cells that have high affinity for peptides are not allowed to mature and do not enter circulation, DCs presenting self-antigens can tolerise T cells in the periphery.

Immature DCs *in-vivo*, contrarily to those *in-vitro*, where high levels of MHC molecules on their surface (Inaba et al., 1998), and tolerance seems to be targeted at peptides presented on immature DCs that do not undergo maturation (Jonuleit et al., 2000). The tolerogenic DC presenting the peptides to T cells in the absence of maturation stimuli may induce T cells to proliferate but these become anergic to further stimulation (Moser, 2003). Alternatively, evidence seems to suggest that immature tolerogenic DCs may induce T cell tolerance to self components by stimulating regulatory T cells (Treg cells) that have a suppressive action (Dhodapkar et al., 2001, Jonuleit et al., 2000, Moser, 2003, Steinman et al., 2003). So far three types of Treg cells have been identified two of which can be induced in an IL10 and/or TGF β dependent way, and a third that arises spontaneously. This mechanism would promote memory of tolerance.

In the construction of a vaccine vector the possibility of inducing tolerance over immunity needs to be taken into account, and possibly eliminated by ensuring a good level of maturation of the cells prior to injection.

1.2.7 Dendritic cells as tools for immunotherapy

Because of their ability to take up exogenous antigens and present them to both CTL and B cells stimulating targeted responses, DCs are the ideal tool in the construction of an immunotherapeutic vaccine vector. Also, the possibility of obtaining large numbers of DCs in culture both from monocytes in the blood (for human cells) and from mouse bone marrow (for murine cells), allows testing for the efficiency of the vector by *ex vivo* loading of the DC, followed by re-injection, comparing it to direct immunisation. A major factor to consider in working towards an immunotherapeutic vector is to ensure correct maturation of the DC. This is both to avoid tolerance and to ensure maximal stimulation of T cells.

Advantages of using *ex vivo* loading include the possibility of monitoring the level of cell maturation, the efficiency of antigen uptake by DC and the dose of cell vaccination, together with the possibility of targeting the antigen-loaded DCs to specific locations, e.g. lymph node or tumour. On the other hand, direct *in vivo* vaccination is a more practical method with reduced manipulation, although specific targeting to the DCs can not be ensured but could still be achieved by cross presentation.

A problem with *ex vivo* antigen loading, is that cell reinjection may result in poor travelling of the DC to the LN. Less than 5% of the injected cells usually reach the draining node (De Vries et al., 2003), implying that specific stimuli such as immunostimulatory cytokines that stimulate maturation and migration should be added to the vaccine. An alternative to override this problem is intranodal injection of antigen presenting cells, but although this is expected to increase T cell stimulation, it may well destroy the structure of the node, forcing the cells to migrate to the next one. Ideally a vaccine would be able to target and activate DCs directly *in vivo*, bypassing the *in vitro* manipulation, but appropriate activation stimuli to obtain correct cell maturation and avoid tolerance should be included (Bonifaz et al., 2002).

The DC targeted by the vector should therefore take up and present the antigen efficiently, as well as being stimulated and allowed to undergo maturation correctly. The stimuli induced by antigen uptake may be enough to activate the

DC, alternatively, the cell may respond to exogenous cytokines (eg. TNF) released by neighbouring lymphocytes.

Numerous methods are available to achieve peptide presentation by the DCs, and all can be used for *ex vivo* or *in vivo* DC targeting although they vary in the strength of the response induced, efficiency of DC targeting and antigen processing, and on the level of safety.

1.2.8 Non viral antigen delivery to dendritic cells

1.2.8.1 Peptide loading

The successful use of irradiated viruses (Lidbury et al., 2000) or parasites (Malik et al., 1991) to induce an immune response in a host proved that *de novo* protein synthesis is not required to achieve peptide presentation by DC. A possibility is therefore to make the cell take up already processed short peptides corresponding to one epitope that can bind directly to the MHC molecules on the surface of the cell. Providing the DC with a synthetic peptide minimises problems due to inefficient protein processing or cell disruption by the antigen-carrying vector. In addition, this method allows an immune response to be specifically induced to the immunodominant peptide of an antigen as well as stimulating the most appropriate cell type (CD8⁺T, CD4⁺T, B cell) by using the identified epitope specific for it.

Adoptive transfer in mice of BMDCs pulsed with a peptide from lymphocytic choriomeningitic virus (LCMV) (Ludewig et al., 1998) or *P. yoelii* CS (Bruna-Romero & Rodriguez, 2001) showed that it is possible to induce a protective immune response using this method and focussing only on one epitope. Recently, Behboudi et al (Behboudi et al., 2004) have shown that similar results can also be obtained by using spleen CD8⁺ or CD8⁻ DC. These cells pulsed with the *P. berghei* CS peptide can induce or boost an IFN γ response to the peptide after i.v. immunisation.

Because of the very low level of safety concerns, this method has also been tested in non human primates where PBMC pulsed with ovalbumin could induce antibody responses in addition to CTL (Barratt-Boyes et al., 1998), and cancer

studies have seen this method tested in humans as well. In a group of seven patients with advanced carcinoembryonic antigen (CEA)-expressing gastrointestinal malignancies four developed CTL responses following immunisation with PBMCs loaded with a CEA restricted peptide (Matsuda et al., 2004). Similarly, three out of four patients with stage IV melanoma receiving PBMCs pulsed with a peptide from MAGE3, usually presented on HLA-A1, showed regression of tumour metastases and increased T cell responses (Godelaine et al., 2003).

A limitation of this loading technique is that only one epitope can be targeted. This has been overcome by loading DCs with a cocktail of epitopes from the same protein, allowing to target cells that facilitate and enhance the response (eg. CD4⁺Th) (Svane et al., 2004). Alternatively a mixture of peptides from different antigens can be used, resulting in a multiantigen vaccine. Combinations of malaria peptides have been administered to monkeys showing multiantigen long lasting B, CD4⁺Th and CD8⁺CTL responses (BenMohamed et al., 2004). Similarly, patients receiving PBMCs pulsed with a mixture of four melanoma antigen HLA restricted peptides resulted in five out of nine expressing a response to the antigens, and three out of these showing a response to two or more antigens (Palucka et al., 2003).

A drawback of this approach remains the laborious effort required to find the immunodominant epitopes and the fact that the peptide-MHC complex formed from extracellular binding is not very stable and dissociates quite rapidly (Banchereau & Steinman, 1998). In addition, for synthetic peptides to be delivered directly *in vivo*, adjuvants are required that may often cause significant inflammatory reactions (Edelman et al., 2002). Recently, *in vivo*, delivery of peptides has been improved by using biodegradable poly-D,L-lactide-co-glycolide (PLGA) microspheres that allow release of antigen over a longer period of time, but this still does not provide an immunogenicity comparable to that of other loading methods (Rosas et al., 2001)

1.2.8.2 DNA/RNA Loading:

DNA/RNA pulsing of DCs means that the DNA/RNA encoding for the gene of interest is directly delivered into the cell. This has the major advantage of allowing the cell to carry out expression and processing of the antigen peptides naturally. Advantages of using DNA loading include the inexpensive production and safety upon injection, as well as the option of using the full-length gene. The use of the entire gene provides additional advantages such as the availability of CD4, B cell, and also additional unknown epitopes. The other advantage of loading a DC directly with DNA is that the regulatory sequences can also be included and expressed, providing the signalling sequences for efficient peptide processing. Moreover the MHC-peptide complex formed from internal processing of the antigen is more stable than that formed from incubation of cells with peptides.

Bone marrow DCs can be transfected *ex vivo*, non-virally, by lipofectin, DNA-liposome complexes, calcium phosphate precipitation, or electroporation. Lately gene gun transfection has been shown to be the most efficient of these methods (Larregina et al., 2004), consisting of gold particles loaded with the DNA of interest that are shot directly into the cytosol of the cell. Cationic peptides are another option, these are positively charged peptides that condense the DNA to allow easier transfection (Irvine et al., 2000). Direct DNA loading of DCs *in vivo* is also possible. The plasmid expressing the specific antigen can be injected directly into the host, but the risk is that this will be taken up by other cells, resulting in cross presentation. Targeting of a DNA vaccine can be improved by including unmethylated CpG sequences, to target the DNA to TLR9-expressing cells (Klinman et al., 1997). Similarly, using other delivery techniques such as the gene gun (Sakai et al., 2003), or microparticles and nanoparticles (Yang et al., 2004) efficiency of delivery to DCs can be improved.

DNA immunisation can induce both B and T cell responses (Leitner et al., 1997), and direct immunisation with naked DNA has proved able to stimulate CTL responses both in mice, monkeys and humans. Gene gun immunisation of a DNA vaccine expressing both the PbCS protein and IL12 into the liver or skin successfully stimulates Th1 type responses (Yoshida et al., 2000). Antibody

responses against malaria proteins have also been generated using this method (Belperron et al., 1999, Haddad et al., 1999, Sakai et al., 1999). In mice, intramuscular injection of naked DNA expressing PyCS resulted in 18 out of 28 mice being protected against subsequent challenge (Sedegah et al., 1994), but in humans, while a response to the malaria PfCS protein could be induced (Le et al., 2000) protection required very high doses of DNA (Wang et al., 1998b).

The success of vaccination relies on the successful transfection and activation of DC followed by initiation of T cell responses. It is difficult for a DNA vaccine to induce maturation whereas *ex vivo* transfected cells can be matured prior to injection. *In vivo* administration of naked DNA should therefore make use of adjuvants or carry signals/genes such as co-stimulatory molecules (eg. CD40L) that will induce DC maturation (Agadjanyan et al., 2003). The main problem with DNA transduction is the inefficiency of gene transfer (Arthur et al., 1997), possibly because of limited transfer to the cell nucleus (Luo & Saltzman, 2000). In addition, entering the nucleus may lead to integration of the DNA. Dendritic cells are therefore often also transduced with RNA. This has similar advantages to DNA but nuclear entry is not required and integration is avoided.

Similarly to DNA, RNA transfection can be achieved by electroporation giving similar levels of delivery as using poxviruses or adenoviruses (see later) (Van Tendeloo et al., 2001), and has the advantage that total RNA, for instance from cancerous cells, can be transfected into mouse BMDC (Jung et al., 2004). Electroporation into DC of the mRNA for the MAGE-A3 cancer antigen, showed that peptides are presented on both MHCI and MHCII (Bonehill et al., 2004). Also, mice immunised with DC transfected with total RNA from tumour cells can stimulate antitumour CTL responses and establish antitumour immunity (Liu et al., 2004). Similarly, human DCs transfected with RNA expressing carcino-embryonic antigen (CEA), have been shown to successfully induce CTL responses (Nair et al., 1998).

1.2.8.3 Bacteria loading:

The use of recombinant organisms that naturally target DCs is an improvement to DNA vaccination since full-length genes can be delivered to the cell for

endogenous processing onto MHCI. Bacteria can infect dendritic cells, resulting in antigens carried by the pathogen being expressed in the DC, and entering the MHCI pathway for presentation. This mechanism inducing CTL responses, makes bacteria an efficient vector for gene delivery to DC. Amongst others, attenuated strains of *Salmonella typhimurium* and *Listeria monocytogenes* have proved efficient in delivering DNA for tumour or viral antigens to DC, resulting in CTL responses and protective immunity (Cochlovius et al., 2002); (Weiskirch & Paterson, 1997).

Safety issues may arise using whole bacteria. Therefore their use has often focussed on bacterial toxins. These can enter the cytosol and traffic in a retrograde manner across the ER into the biosynthetic and secretory pathways. Fusion of a DNA antigen sequence to a bacteria toxin sequence allows for entry into the dendritic cell using the pathway usually used by the bacteria. Bacteria and anthrax toxins can enter directly through the endosomal membrane whereas the *Bordatella pertussis* enters via the plasma membrane (Moron et al., 2004). This then allows expression of the antigen within the DC so that it will undergo MHCI presentation. Adenylate cyclase (CyaA) derived from *B. pertussis* efficiently binds to the surface of DCs and can therefore specifically target these cells for the delivery of genetic material. CyaA coupled to viral or tumour epitopes has been shown to induce protective or therapeutic responses (El Azami El Idrissi et al., 2002, Fayolle et al., 1999).

1.2.8.4 Virus Like Particle (VLP) Loading:

Virus like particles are formed by viral structural proteins that retain the virus external capsids but not the whole genome. Proteins on the surface of a virus are usually responsible for the entry into cells, therefore VLPs maintain this ability to enter cells and release the antigenic material that they contain while not undergoing replication. VLP have the advantage that they are able to stimulate DC maturation with the production of costimulatory signals to ensure the generation of efficient immune responses. They are, therefore, natural adjuvants, and induce a long lasting immune response (Sedlik et al., 1997).

VLP can be formed based on a number of viruses. One of the most widely used VLP is Hepatitis B virus small envelope protein (HBsAg), although papillomavirus (Chackerian et al., 2004) and parvovirus (Moron et al., 2003) VLPs have also proved efficient. Chimeric VLPs can carry foreign peptides and induce B and T cell responses following DC internalisation by micropinocytosis. HbsAg relies on endosomal processing in DCs. The antigen is processed inside the endocytic vesicle without reaching the cytoplasm, therefore binding to pre-existing MHCI molecules. Other VLPs may be TAP independent (Ruedl et al., 2002) or TAP dependent (eg. parvoviruses) (Moron et al., 2003).

Ty-virus like particles (Ty-VLP) consist of a single protein species that can be produced in yeast. They have been used for malaria immunotherapy expressing a string of 15 CTL epitopes from *p. berghei* resulting in non-protective responses following a single immunisation, though suggesting efficient targeting of DC (Gilbert et al., 1997). Similarly, clinical trials using Ty-VLP carrying part of the HIV1Gag gene (p24-VLP) gave disappointing results (Lindenburg et al., 2002). Although not strong enough to induce protective responses, Ty-VLPs have been found to act as good priming agents for subsequent viral boost (Gilbert et al., 2002, Oliveira-Ferreira et al., 2000).

1.2.9 Viral vectors for immunotherapy:

Viruses are ideal for the delivery of genetic material to cells since they have evolved to gain access to cells and to use the cell machinery to facilitate their own replication. This is therefore probably the most efficient way of introducing exogenous DNA directly into the nucleus of DCs allowing natural processing and presentation of the peptides, resulting more efficient than other techniques (Larregina et al., 2004). Similarly to other DNA loading methods, expression of large amounts of DNA is possible, and superior antigen processing with more sustained presentation is seen compared to peptide uptake (Brossart et al., 1998, Germain & Margulies, 1993). In addition, viruses are usually relatively easy to propagate and to grow at high titres.

An advantage unique to viruses, in particular retroviruses and lentiviruses, is that pseudotyping of the envelope can overcome the limitation of a virus not naturally

infecting DC. Alternatively, molecules (eg. CD40L) that will target the virus directly to the DC can be expressed on the coat of the virus. When using the virus for direct *in vivo* injection, specificity of infection is particularly important since the gene might be expressed in non-target cells. However, even here infected cells may be taken up for cross-presentation. Using *ex vivo* transduced DCs is in this case, therefore, an advantage.

A concern when using viral vectors, is safety and toxicity of the virus. The challenge in viral vector therapy is to maintain the efficiency of infection while minimising toxicity. Ideally the vector should induce an immune response only against the recombinant gene. Virus replication in DCs depends on the virus, and in some cases on the maturation state of the cell (Kaiserlian & Dubois, 2001). Lentiviruses and rAAV are only minimally immunogenic in humans, therefore have good potentials as vectors for gene delivery.

An important point that needs to be addressed when using viruses to deliver antigens to DCs, is the effect that infection might have on cell functions. HSV-1 and vaccinia virus, for example, when infecting immature DCs prevent the cell from undergoing subsequent maturation. Influenza or measles virus, on the other hand, allow DC maturation. The ability of the cell to mature and present antigens may be impaired as a result of genes expressed by the virus as an immune evasion mechanism. Deletion of such genes is therefore necessary to ensure efficient gene delivery and expression. Toxicity may also be avoided by ensuring efficient expression such that a lower dose of virus is required. At the same time, viral pathogenicity may act as a form of adjuvant.

Injection of a virus will stimulate an immune response even if highly attenuated. It is important to keep this response to the minimum to avoid destruction of the vector prior to stimulation of an immune response. Similar problems may arise from previous exposure of the host to the virus. *Ex vivo* transduction of DCs can again ensure that the virus has entered the cells rather than being eliminated by immune responses before reaching them *in vivo* (Brossart et al., 1997).

Finally, an advantage of using viruses is that, depending on the virus, it is possible to incorporate relatively large amounts of exogenous DNA (Table 1.3). The deletion of toxic and non-essential genes helps in further increasing the amount of foreign DNA that can be incorporated. There are five main groups of viruses that have been considered for gene therapy: adenoviruses, adenoassociated viruses, retroviruses, lentiviruses and herpesviruses. Contrarily to transduction with naked DNA that is extrachromosomal, viruses can be further subdivided into extrachromosomal DNA viruses that persist in the infected cells as an episome (adenoviruses, adeno associated virus, herpesviruses), or integrating viruses (retroviruses) which may cause a further problem with insertional mutagenesis.

Viral vector	Insert size	Advantage	Disadvantage
Adenovirus	8Kb	Broad target population	Immunogenicity
Adenoassociated	4Kb	Non immunogenic	Small insert size
Lentivirus	8Kb	Long term expression	Insertional mutation
Retrovirus	8Kb	Long term expression	Only dividing cells Insertional mutation
Herpes simplex virus 1	30-40Kb	Large inset size	Very immunogenic

Table 1.3 Main characteristics of five principally used viral vectors

1.2.9.1 Adenovirus:

Adenoviruses are double stranded DNA viruses with the ability to infect both dividing and non-dividing cells. Adenoviruses preferentially infect immature DCs (Zhong et al., 1999), with the advantage of not interfering with cell maturation (Dietz et al., 2001), although at high MOI DC viability is reduced (Rea et al., 1999, Rouard et al., 2000), and that the transferred genetic material is epichromosomal (Ehrhardt et al., 2003).

Adenoviruses allow delivery of only up to ~8Kb of exogenous DNA depending on the number of deleted genes. However, the main problem with adenovirus vectors relates to their immunogenicity. Expression of the adenoviral early genes in DCs arrests the cell cycle of the stimulated T cells (Tuettenberg et al., 2004). Replication incompetent adenoviruses deficient in immediate early genes (E1 and E3) have therefore been used, but these still present with the possibility of reforming replication competent viruses. Further attenuated adenoviral vectors have been engineered leading to a “gutless” version that has all viral coding

sequences removed and relies on a helper virus for replication. These viruses allow for long term expression, insertion of up to 37Kb of exogenous DNA and preparation of high titre stocks (Zou et al., 2000). The possibility of growing high titre stocks is important for this type of virus since high titres are needed to achieve good levels of transduction in DCs (Linette et al., 2000, Rea et al., 1999, Zhong et al., 1999).

Of the 51 different serotypes, serotypes 2 (Ad2) and 5 (Ad5) have mainly been studied, initially used to target the respiratory epithelium in cystic fibrosis studies (Kovesdi et al., 1997). These have then been further developed as gene delivery vectors to target other tissues and cells, including DC. Infection occurs by initial binding of the a capsid protein to the coxsackievirus and adenovirus receptor (CAR) on the cell surface (Bergelson et al., 1997, Tillman et al., 1999). Further interactions of proteins with integrins on the host cell surface result in virus internalisation (Barnett et al., 2002, Mathias et al., 1994, Wickham et al., 1993). Targeting can be improved by addition of a cell binding motif or CD40L in the capsid, and this has been shown to increase infectivity of DC and recombinant gene expression (Stockwin et al., 2002, Tillman et al., 1999, Worgall et al., 2004, Zhang et al., 2003).

Genetically modified adenoviruses can induce both cellular and humoral immunity. This virus has been used in vaccine studies including cancer, viral and parasitic infections. Mice immunised with dendritic cells infected with adenovirus expressing the MART1 antigen could stimulate CTL responses and confer partial protection (Broder et al., 2003), and studies using the thyroid peroxidase gene additionally showed stimulation of antibody responses following injection with infected DCs or neat adenovirus (Guo et al., 2003). Similarly, in malaria research, an adenovirus expressing PyCS could successfully stimulate CD8⁺ and CD4⁺T cells upon virus injection implying that DCs are targeted and can present the recombinant antigen to induce protection (Rodrigues et al., 1997, Rodrigues et al., 1998). Non-human primates have also received DC transduced with recombinant virus expressing the Gag antigen of SIV, and successfully developed CD8⁺T cell responses following skin or lymph node injection (Brown et al., 2003)..

1.2.9.2 Adeno-associated virus (AAV):

AAV is a non-pathogenic single stranded DNA parvovirus with a genome less than 5Kb long that can accomodate 4Kb of exogenous DNA. AAV needs a helper virus to replicate (Muzyczka, 1992). Wild type AAV specifically integrates on chromosome 19, although vector viruses integrate non-specifically. AAV only expresses two sets of proteins: replication proteins (*rep*) and capsid proteins (*cap*). The *rep* proteins are toxic due to downregulation of several cellular promoters. Most of the AAV regulatory elements (packaging, integration) are included in the inverted terminal repeats (ITRs) making it possible to delete both *rep* and *cap* proteins, replacing these with the genes to be delivered, and exploit the ITRs for replication, integration and packaging.

AAV is capable of infecting numerous human cell types both *in vitro* and *in vivo*, giving long term gene expression without requiring cell division. This makes AAV a safe vector to target various organs (Ponnazhagan et al., 2001). In particular, AAV can infect both murine and human dendritic cells that remain unaffected in their ability to undergo maturation (Liu et al., 2000b, Ponnazhagan et al., 2001, Zhang et al., 2000).

AAV was initially identified as a possible vector to deliver and express genes for muscular dystrophy inducing strong cellular and humoral immune responses (Cordier et al., 2001). *In vitro*, rAAV has been shown to deliver viral (Chiriva-Internati et al., 2002, Sun et al., 2002) and tumour (Chiriva-Internati et al., 2003) antigens to human dendritic cells with stimulation of both CD4⁺ and CD8⁺ T cell responses. Similarly, *in vivo*, injection of recombinant AAV expressing HSV-2 glycoprotein B and D could stimulate both humoral and cellular responses (Manning et al., 1997), as did transfer of BMDC infected with a LacZ recombinant AAV leads to same type of responses (Zhang et al., 2000).

Although AAV is non-pathogenic, making it a safe option for use in humans, more than 80% of the population has antibodies against at least one AAV serotype, reducing its efficiency as a vector in these people (Peden et al., 2004).

1.2.9.3 Retrovirus:

Retroviruses are enveloped ssRNA viruses that infect cells by binding to specific receptors on the cell surface. Their disadvantage of being poor at infecting non dividing cells can easily be overcome by pseudotyping. Vesicular stomatitis virus protein, for example allows for efficient transduction in the majority of dividing cells.

Following infection of a cell with a retrovirus, the incoming RNA is reverse-transcribed and randomly integrated into the host genome. This is an advantage as it allows the long term presence of the recombinant gene, though not ensuring stable expression, although insertional mutagenesis is also possible.

Moloney murine leukaemia virus (MMLV) is a typical retrovirus, containing the three genes identifying retroviruses: *gag* (structural protein), *env* (envelope protein) and *pol* (for nucleic acids metabolism). Additionally, the LTRs contain signals for reverse transcription, integration and transcription. Retrovirus based vectors can be constructed by transfecting vector plasmids that contain the gene of interest (up to 8Kb) flanked by the LTRs and *cis*-elements into packaging cells that provide, *in trans*, the other proteins.

Although retroviral vectors are inefficient at infecting non dividing cells, dividing DC progenitors have been shown to be transfected by these viruses and retain their ability to differentiate into DCs capable of inducing T cell activation (Bello-Fernandez et al., 1997, Grignani et al., 1998, Szabolcs et al., 1997). Using dividing progenitor cells, retroviruses have therefore been proved efficient in stimulating responses to tumour associated antigens (HER2) (zum Buschenfelde et al., 2001), parasite infection (PySSP2) (Hettihewa, 2003), as well as responses against bacteria (*Lysteria monocytogenes*) (Nakamura et al., 2003) or viral antigens (HbcAg) (Ding et al., 2003).

Despite the safety concerns of using retroviruses, CD34⁺ cells transfected with a Murine Leukaemia virus (MLV) based vector have been tested in clinical trials for

human X-linked severe combined immunodeficiency resulting so far already in three cases of adverse reaction (Buckley, 2002, Cavazzana-Calvo et al., 2000).

1.2.9.4 Lentivirus:

Lentiviruses are retroviruses that, in addition to *gag*, *env* and *pol*, express accessory proteins that contain nuclear localisation factors, allowing for transduction of non-dividing cells (Emerman, 1996, Lewis & Emerman, 1994). Both blood derived human DC or mouse bone marrow derived DC, which are preferentially immature, can be transduced (Gruber et al., 2000, Negre et al., 2000). However, it appears that transduction of dividing human CD34+ progenitor cells is the most efficient (Salmon et al., 2001). In addition, maturation is not affected but rather stimulated by the presence of the virus (Esslinger et al., 2002, Gruber et al., 2000).

Lentivirus derived vectors are mainly based on human (HIV) or simian (SIV) immunodeficiency virus. They can be easily attenuated by deletion of genes, and can deliver up to ~8Kb of foreign DNA. As retroviruses, they integrate their provirus in certain non-dividing cells allowing long term persistence in DCs (Lewis & Emerman, 1994). High titre stocks of lentiviral vectors can relatively easily be produced, and an advantage of lentivectors is that pre-existing immunity in the host is relatively rare. In addition, immunogenicity of these viruses is low even though they are able to induce stronger immune responses to the delivered antigen than are adenovirus transduced cells (Esslinger et al., 2002).

Safety reasons require that lentiviral vectors are highly attenuated. Highly attenuated HIV-1 based vectors expressing the Flu peptide could efficiently transduce immature DCs of which 40% retained their ability to express the gene and the ability to activate T cells following maturation (Dyall et al., 2001). Also, highly attenuated SIV-based vector can efficiently transduce both terminally differentiated and monocyte-derived human DCs, suggesting that vectors based on lentiviruses from other species than humans could be used to reduce safety concerns (Mangeot et al., 2000) .

To avoid formation of infectious particles, recombinant lentiviruses are packaged in permanent, inducible packaging cell lines that express *gag*, *pol*, *rev* and the VSV-G envelope gene. The gene of interest is then introduced by repeated transduction by lentiviral particles prepared by transient transfection.

DCs transduced with lentivectors can stimulate immune responses against viruses, or melanoma (Metharom et al., 2001) upon injection of mice, and this response appears to be stronger than obtained with peptide pulsed (Esslinger et al., 2002, Zarei et al., 2004), or adenovirus transduced DCs (Esslinger et al., 2002). Moreover, efficiency of cell maturation and T cell responses have been further improved by including the expression of CD40L (Koya et al., 2003).

1.2.9.5 Vaccinia virus:

Vaccinia virus is a double stranded DNA viruses that remain extrachromosomal. Vaccinia virus has the advantage of allowing the delivery of multiple genes (up to 25Kb can be delivered) (Carroll & Moss, 1997). Vaccinia virus does not replicate within DCs, making it a safe vector (Jenne et al., 2000). The two highly attenuated strains of vaccinia virus which are most commonly used are NYVAC (NewYork vaccinia strain), and the MVA (Modified Vaccinia Ankara). These transduce DCs even at low MOIs (up to 60% at MOI 2.5) (Drillien et al., 2000, Sutter & Moss, 1992), and induce maturation upon infection (Bonini et al., 2001, Engelmayer et al., 1999); (Drillien et al., 2004). Both immature and mature DC can be transduced by endocytosis (Drillien et al., 2000). However, wild type virus does not induce DC maturation (Jenne et al., 2000). Infection, instead, results in a decrease in CD83 and CD80 expression on the cell surface paralleled by inhibition of maturation of immature cells and decreased ability to stimulate T cell responses from mature cells (Drillien et al., 2000, Engelmayer et al., 1999, Jenne et al., 2000).

Recombinant vaccinia viruses have proved relatively safe and effective. Following a DNA or an adenovirus priming dose, an MVA boost confers increased and protective CD8⁺, CD4⁺ and antibody responses to the malaria CS protein in mice (Bruna-Romero et al., 2001); (Gilbert et al., 2002). Similarly MVA has been applied to cancer therapy showing a response against MUC1 or

tyrosinase after a single immunisation (Drexler et al., 1999, Trevor et al., 2001), and is now being developed as a vaccine for HIV (Nkolola et al., 2004), human cytomegalovirus (Wang et al., 2004), and also HSV-2 (Meseda et al., 2002). Norbury et al have shown the presence of infected DCs in the draining LN following virus infection thus supporting the use of this virus for direct injection (Norbury et al., 2002)

Human clinical trials have taken place targeting DCs in vivo for malaria therapy following DNA priming showing strong CD4⁺ and CD8⁺ responses (McConkey et al., 2003, Moorthy et al., 2003b). MVA can provide both a good initial stimulus and boosting dose, a boosting regime composed of two different poxviruses can lead to strong CTL responses in humans (Moorthy et al., 2004b), including when using multiple antigens (Prieur et al., 2004).

The delivery of multiple genes, has proved efficient in non-human primates, where DNA and virus expressing the malaria CS, SSP2, AMA1, and MSP1 antigens resulted in protection of 2 out of 11 monkeys, and 7 were able to resolve parasitaemia spontaneously (Rogers et al., 2002).

1.2.9.6 Influenza virus:

Influenza virus is a negative stranded RNA virus that has the advantage of safety from the point of view of integration into the genome of the host cells. Mature human monocyte derived DCs can be transduced very efficiently resulting in more than 90% of cells containing the virus at MOI1, as shown by expression of the tumour associated antigen MAGE3 (Strobel et al., 2000). The infected cells retain their ability to mature, do not undergo apoptosis and can generate strong antiviral CD8⁺T cell responses but only at low MOI even if the virus used is inactivated (Bender et al., 1998). In addition, DCs have also been shown to cross-present on MHCI viral proteins from other cells infected with influenza virus (Albert et al., 1998).

The possibility of using influenza virus for the induction of immune responses was first shown by Li et al using a recombinant virus expressing an epitope of the PyCS protein to stimulate T cell responses to plasmodium (Li et al., 1993).

Dendritic cells infected with attenuated influenza virus have now been shown to induce CTL responses also to tumour genes (Strobel et al., 2000) and proto-oncogenes (Efferson et al., 2003).

1.3 Herpes Simplex Virus 1 (HSV-1)

1.3.1 Pathology of HSV-1

Infection with herpes simplex virus 1 (HSV-1) affects over 80% of the population. HSV-1 infects both dividing (eg. epithelial) and non-dividing (eg. neurons) cells. HSV-1 enters the body by infection of epithelial cells of the skin or mucosal membrane, where it replicates at the site of infection. Progeny particles spread to axons of sensory neurones that innervate the primary site of infection and are carried by retrograde axonal transport to neuronal cell bodies where the virus enters latency. Upon occasional reactivation, due to stress, exposure to UV irradiation, tissue damage, or immunosuppression, HSV-1 travels down the neurones and infects the innervating skin mucosa causing the common cold sore.

Although the clinical signs of HSV-1 reactivation are usually mild (cold sore being the most common), in new-born and immunocompromised individuals this infection can lead to fatal encephalitis. Herpetic interstitial keratitis is another pathology that may follow HSV-1 infection, probably caused by tissue damage incurring from a dysregulated inflammatory response (Streilein et al., 1997).

1.3.2 Structure of the HSV-1 virion

HSV-1 is a member of the Herpesviridae family, composed of eight members: Herpes simplex virus 1 and 2 (HSV-1, HSV-2), cytomegalovirus (CMV), Epstein-Barr virus (EBV), varicella zoster virus (VZV), and human herpesvirus 6, 7, 8 (HHV). Within this family, HSV-1 is a member of the alphaviruses group. These are the neurotropic subfamily of herpesviruses. HSV-1 has a double stranded 152Kb DNA genome encoding ~84 proteins, and is the herpesvirus that has mainly been used as gene delivery vector. All herpesviruses have a common genome structure, and some genes that are essential for replication are common between all the members of the family. The genome is organised so that a unique long (UL) and unique short (US) region, are flanked by two pairs of inverted terminal repeats (b/TRL-b'/TRL and c/TRS-c'/TRS). Finally, both ends of the genome contain a short sequence (a) which may be repeated several times containing essential packaging signals (figure 1.8).

The viral genome is enclosed in an icosahedral nucleocapsid separated from the lipid bilayer envelope by a layer of proteins called the tegument, involved in facilitating the early stages of infection and initiation of viral transcription by carrying proteins necessary for these processes. Two of these proteins are virion protein 16 (VP16) that contributes to the activation of immediate early genes, and the virion host shutoff protein (vhs) that favours viral replication by interfering with host protein synthesis (Fink & Glorioso, 1997). The envelope contains eleven glycoproteins (gB-gM) that mediate viral attachment and entry into the cell through fusion of the envelope with the cellular membrane, and it has recently been suggested that the cytoplasmic tails of glycoproteins in the envelope act to anchor the virion envelope onto the tegument (Farnsworth et al., 2003, Gross et al., 2003).

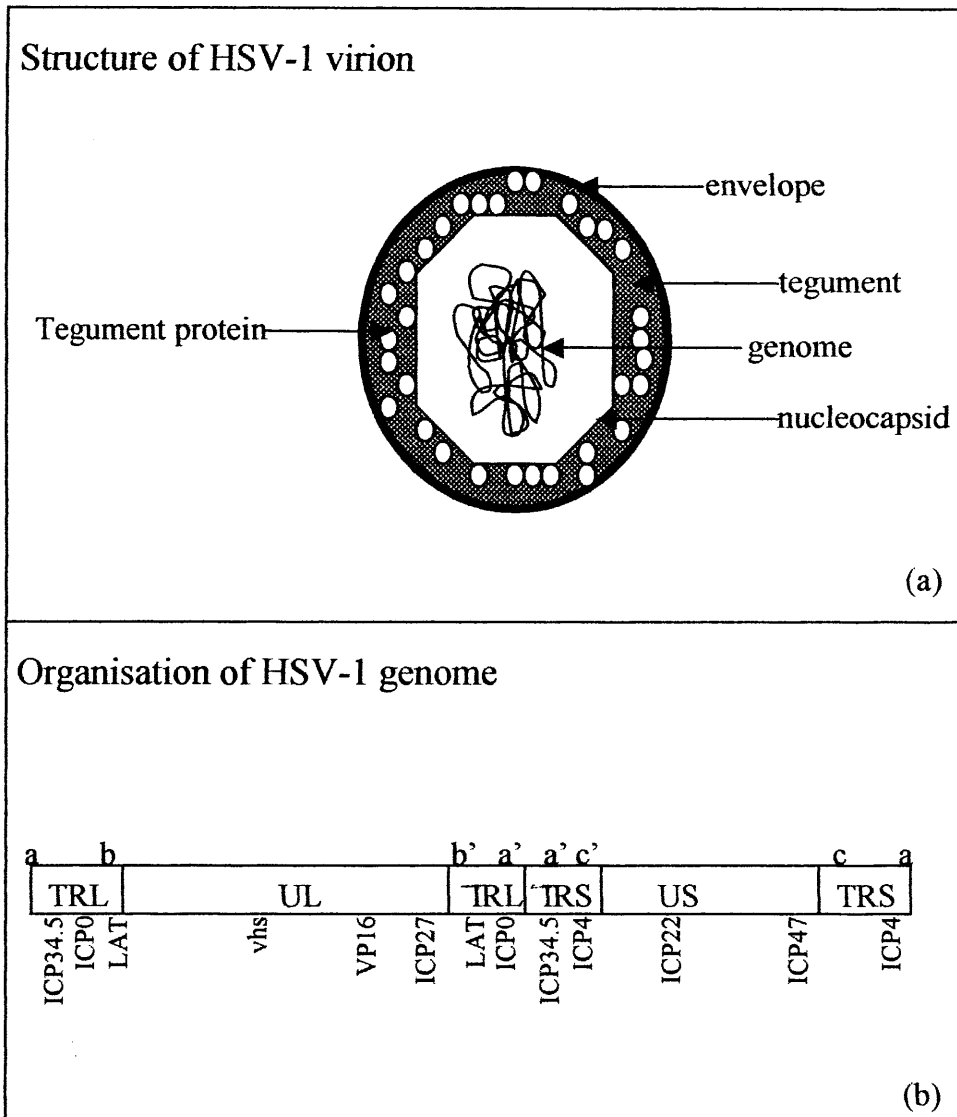


Figure 1.8 HSV-1 structure

(a): Schematic representation of HSV-1 virion structure consisting of double stranded DNA genome enclosed in a capsid and tegument surrounded by an envelope.

(b) Schematic representation of the organisation of HSV-1 genome consisting of unique long (UL) and unique short (US) regions flanked by the terminal long and short repeats (TRL, and TRS) and internal long and short repeats (IRL, and IRS). The position of some most relevant genes is indicated.

1.3.3 Life cycle of HSV-1

HSV-1 enters a cell by interaction of glycoproteins on the viral envelope with receptors on the cell surface leading to membrane fusion. The fundamental viral glycoproteins for this process are gB, gH, and gL, and the cellular receptors involved are a herpesvirus entry mediator (HVEM) receptor, and nectin receptors 1 and 2. HVEM is a member of the TNFR family, found on epithelial cells, fibroblasts, leukocytes, and lymphocytes, including DC. Because of its presence on the surface of intact epithelium, it is believed to be associated with the incoming virus. Nectin receptors 1 and 2, on the other hand, are members of the immunoglobulin family, expressed on epithelial cells, fibroblasts, and neurones (Haarr et al., 2001, Mata et al., 2001). They are thought to be more associated with spreading of the virus within the epithelium and in neurones (Haarr et al., 2001). Initially, gB and gC interact with *binding* receptors in a reversible manner. This gathers viral particles on the cell surface (Shukla et al., 2000). Subsequently, gD binds to *entry* receptors forming permanent bonds leading to membrane fusion possibly by undergoing conformational changes that allow it to interact with gB or with the gH-gL complex that might then trigger fusogenic activity. Alternatively, gD may not be part of this process making binding of gB and/or gH-gL to receptors directly trigger membrane fusion (Spear & Longnecker, 2003).

Following envelope fusion, the tegument and nucleocapsid are released into the cell together with the already formed tegument proteins (principally VP16 and vhs) needed for immediate use in replication. Depending on the cell-type infected, the virus can either undergo lytic replication leading to cell death and virus release, or enter latency, a state in which the virus undertakes only minimal gene expression, and can persist for the lifetime of the host with occasional reactivation.

1.3.3.1 Lytic replication cycle:

Upon initial viral entry, and also after reactivation, the virus can replicate in epithelial cells. Entry of HSV1 into the cells correlates with a shutoff of cellular protein synthesis in favour of viral replication that takes place in a cascade like fashion. Replication starts with expression of immediate early (IE) α genes that

have regulatory functions and are responsible for activation of early (E) β genes involved in DNA synthesis, that in turn initiate late (L) γ genes expression, these further divided into $\gamma 1$ and $\gamma 2$ mainly coding for virion components (figure 1.9). $\gamma 2$ genes promoters have a TATA box but lack regulatory elements making their expression, unlike $\gamma 1$ genes, dependent upon DNA replication (Rajcani et al., 2004).

The VP16 and vhs proteins incoming from the tegument are the two first and key proteins to act in favour of viral transcription. The tegument protein VP16 triggers the replication cascade. VP16 is one of the at least 15 tegument proteins that serve multiple functions at different steps during viral replication. VP16 is a 65kDa protein encoded by UL48 and initially it functions as a transactivator of IE genes although also functioning as a modulator of the UL41 gene product vhs later in the cycle (Lam et al., 1996), and also being required for proper viral assembly and egress (Mossman et al., 2000b). VP16 initiates viral transcription by inducing formation of the activation complex on the α gene promoters that encode for the five immediate early infected cell polypeptides (ICP): ICP0, ICP4, ICP22, ICP27, ICP47.

The C terminal of VP16 corresponds to the transactivator domain and binds to the TAATGARAT sequence present in all promoters of IE genes (Preston et al., 1988). Because VP16 only binds weakly to DNA the cellular Oct1 transcription factor initially binds to the promoter while the host cell factor (HCF) acts as mediator by binding to VP16 and targeting it to the DNA-bound Oct1 for stable interaction with DNA (Herr & Cleary, 1995). This multicomponent protein-DNA binding transcription regulatory complex, called the VP16-induced complex (VIC), initiates transcription of the IE genes that subsequently engage in a self-sustaining cascade (Figure 1.9). In non permissive cells it is possible that the VP16 complex is possibly prevented from binding to the promoters of the IE genes (Latchman, 1999).

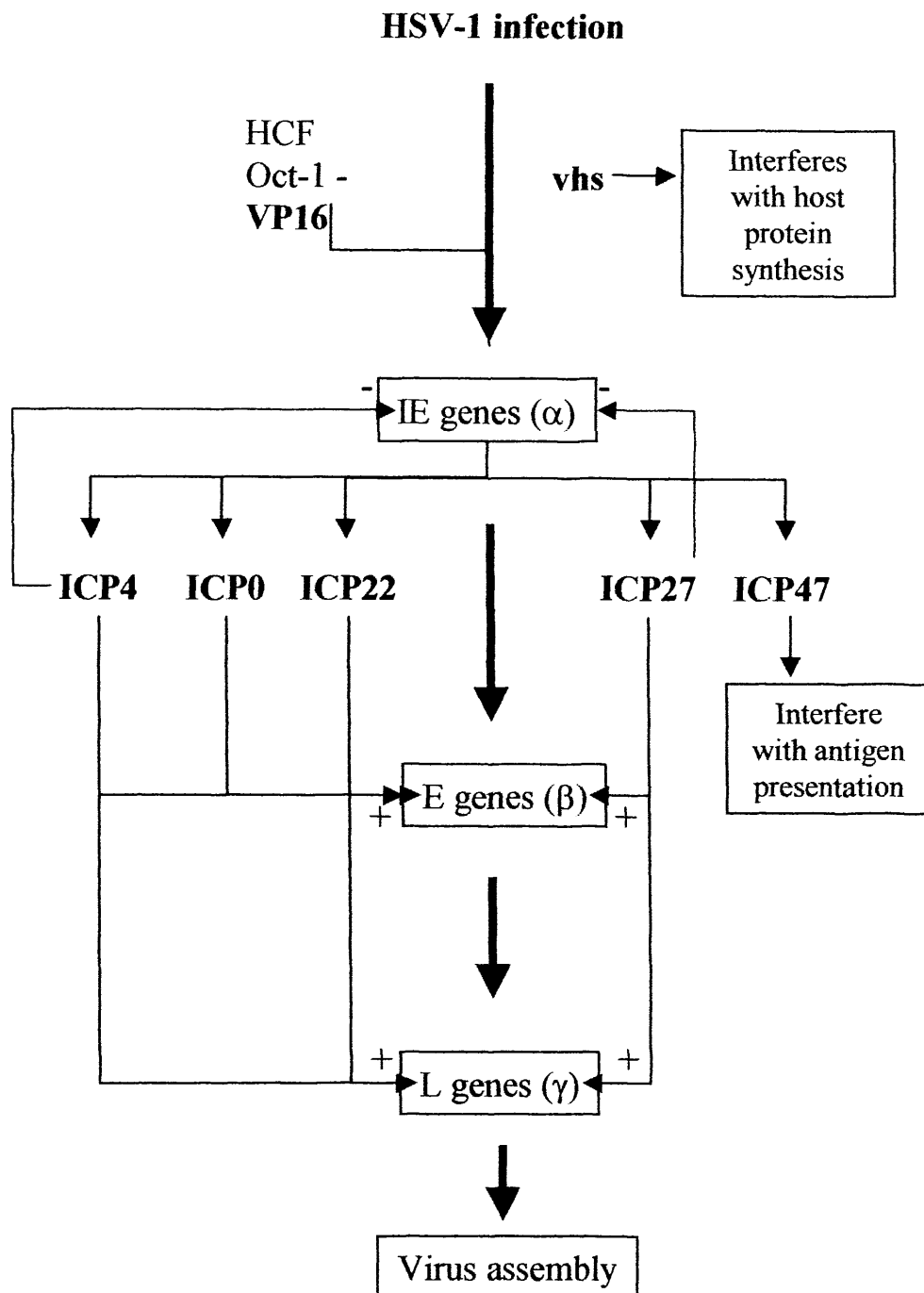


Figure 1.9: HSV-1 lytic cascade

Representation of HSV-1 gene expression during productive infection. VP16 triggers the lytic cascade allowing expression of immediate early (IE, α) genes that activate early (E, β) genes that in turn activate late (L, γ) genes. ICP4, ICP27 and ICP22 act both on E and L genes. ICP4 and ICP27 also downregulate IE genes expression (red arrows)

Of the five immediate early genes ICP4 and ICP27 are essential for viral replication (DeLuca et al., 1985, Sacks et al., 1985) whereas deletion of ICP0 and ICP22 significantly reduces viral growth especially at low MOI (Cai & Schaffer, 1992, Chen & Silverstein, 1992, Sears et al., 1985).

Following their synthesis in the cytoplasm, ICP4 and ICP27 migrate into the nucleus where they form a complex with cellular nuclear structures known as ND10 that are found associated with the parental viral genome. Formation of this new complex with ICP4 and ICP27 is ICP0 dependent at low MOI (Everett et al., 1997, Everett et al., 1998), but not at high MOI (Everett et al., 2004). The complex targets the promyelocytic leukaemia (PML) component of ND10 for destruction in proteasomes and later develops into replication compartments for the expression of early genes (Everett et al., 2004).

ICP0 is then responsible for activation of a few β genes, but its main role is to maintain expression of α genes, acting more as an enhancer to ICP4, specifically efficient at activating β genes (Everett, 1984, Everett, 1987). ICP4 is an important regulator able to transactivate β and γ genes expression as well as being able to downregulate α genes. When phosphorylated, ICP4 binds the promoters with transcription activation complex and cellular factors to stimulate β gene expression, but in its non-phosphorylated form ICP4 downregulates ICP0 and its own expression (Roberts et al., 1988). Furthermore, in conjunction with the action of ICP0 it can also downregulate latency promoters (Batchelor & O'Hare, 1990, Goins et al., 1994). Finally, ICP4 can inhibit expression of β and γ genes before the onset of viral replication. Elimination of ICP4 (essential) results in dramatic decrease in replication, but the virus still induces toxicity in the infected cells despite only four immediate early genes and ICP6 (large subunit of ribonucleotide reductase) and ORF P are produced (DeLuca et al., 1985, Yeh & Schaffer, 1993).

ICP27 is the other gene necessary for the generation of infectious viral progeny (Sacks et al., 1985). ICP27 facilitates DNA replication by enhancing expression of viral factors required for DNA synthesis and interacting with ICP4 (Uprichard & Knipe, 1996). Similarly to ICP4, ICP27 stimulates E and L genes (Samaniego et

al., 1995) while also downregulating IE and E genes (McCarthy et al., 1989, Rice & Knipe, 1990, Sacks et al., 1985). ICP27 in addition acts at the protein level. It collaborates with vhs in the decrease of the amount of mRNA in the cell (Hardwicke & Sandri-Goldin, 1994, Song et al., 2001), and can modulate pre-mRNA polyadenylation efficiency (McGregor et al., 1996, McLauchlan et al., 1992). ICP27 also binds and stabilises the 3' end of the mRNA (Brown et al., 1995), inhibits cell protein synthesis by sequestering snRNPs (Hardwicke & Sandri-Goldin, 1994, Hardy & Sandri-Goldin, 1994) and inhibits pre-mRNA viral splicing as well as allowing retention in the nucleus of transcripts still containing introns (Hardy & Sandri-Goldin, 1994); (Phelan et al., 1996). By inhibiting splicing of mRNA (McCarthy et al., 1989, Sacks et al., 1985) ICP27 establishes a method of stopping only translation of cellular genes, as only very few viral genes contain introns.

ICP22 is a cytotoxic protein that activates β and γ genes by a phosphorylation mechanism including cellular RNA polymerase II (Rice et al., 1995). Deletion of ICP22 results in delayed early genes transcription and reduced late genes activation (Sears et al., 1985).

Finally, ICP47 is responsible for blocking peptide presentation on MHCI molecules by interfering with the activity of the protein transporter associated with antigen processing (TAP) by binding to the substrate binding site (Jugovic et al., 1998, Neumann et al., 1997). Deletion of ICP47 does not impair viral growth (Mavromara-Nazos et al., 1986).

Vhs is mainly responsible for the destabilisation of cellular mRNA and inhibition of host protein synthesis following infection (Fenwick & McMenamin, 1984, Kwong & Frenkel, 1987). Vhs is found in all alphaherpesviruses sequenced so far, synthesised as a γ gene, but acting as an α gene. It has a sequence very similar to that of cellular nucleases involved in DNA replication and repair. In fact, mutations that disrupt the catalytic activity of its homologues also disrupt the activity of vhs (Doherty et al., 1996, Everly et al., 2002, Everly & Read, 1997).

Vhs mainly acts with ICP27 to decrease the amount of mRNA in the cytoplasm without allowing de-novo synthesis. It is not clear whether vhs is an RNase or if it is part of a complex, but it acts as an mRNA specific RNase both increasing the rate of mRNA degradation in the cytoplasm and by stopping cell protein synthesis (Kwong & Frenkel, 1987, Strom & Frenkel, 1987). Vhs disrupts pre-existing polyribosomes (Laurent et al., 1998), and helps ICP27 in inhibiting pre-mRNA splicing (Hardy & Sandri-Goldin, 1994) and repressing primary transcription (Long et al., 1999, Rice et al., 1995, Spencer et al., 1997).

Both cellular and viral cytoplasmic mRNAs are degraded, and both 5' and 3' ends of the mRNA are targeted (Elgadi et al., 1999, Karr & Read, 1999, Kwong & Frenkel, 1987, Oroskar & Read, 1987, Oroskar & Read, 1989). Initial studies looking at HSV TK suggested that vhs targets the 5' sites of initiation of translation since this was degraded before the 3' end (Karr & Read, 1999), and in addition, vhs has been shown to bind directly to cellular translation initiation factors like eIF4H (Feng et al., 2001). However, recently vhs has been suggested to be targeted at 3' end AU-rich elements (ARE) that promote deadenylation and decapping in uninfected cells, followed by 3'-5' degradation (Esclatine et al., 2004a; Gao et al., 2001, Lai et al., 2003; Taddeo et al., 2003). Upon infection some mRNAs are induced and not degraded, amongst these are mRNAs encoding for ARE-targeted proteins (ex TTP, TIA1 and TIAR) all of which involved in degradation of ARE containing mRNAs (Esclatine et al., 2004b). Infection with vhs- virus result in lower amounts of TTP in the cytoplasm so, although vhs possibly does not upregulate TTP expression, in infected cells the degradation of ARE containing RNA is vhs dependent. TTP interacts with vhs to initiate 3' degradation with a similar mechanism to the one that takes place on ARE containing RNAs in non infected cells (Esclatine et al., 2004a, Taddeo et al., 2003).

Vhs mutated viruses only undergo a 5-10 fold reduction of replication in culture (Read & Frenkel, 1983, Read et al., 1993, Smibert & Smiley, 1990) although *in vivo* replication is severely impaired, probably due to the role of vhs in immune evasion (discussed in 1.3.4.2) (Leib et al., 1999, Strelow et al., 1997, Strelow & Leib, 1995). In neurones, vhs deficient viruses show a significant reduction in

growth (Smith et al., 2002, Strelow & Leib, 1996), and vhs has been shown to degrade mRNA in these cells but requires much higher MOI. Susceptibility to vhs may therefore be reduced by nerve growth factor differentiation (Strand et al., 2004).

VP16 interacts with the lytic cycle not only by initiating it but also by supporting it later in infection by binding and inhibiting vhs activity to promote viral protein synthesis (Lam et al., 1996, Smibert et al., 1994; Knez et al., 2003).

1.3.3.2 Latent cycle:

Alternatively to the lytic cycle, the newly released particles can infect nearby sensory neurones present at the site of infection. The nucleocapsid is then transported in a retrograde manner to the cell body in the ganglia and enters the nucleus. Neurones may become productively infected and spread the virus across synapses resulting in death of the cell. Alternatively, HSV-1 can persist for a lifetime in the latent form as a stable episome (Gilden et al., 2001, Hemmi et al., 2002). Latency is usually established in trigeminal ganglia or sacral dorsal root ganglia but also in the CNS (Baringer & Pisani, 1994, Itzhaki et al., 1997, Liedtke et al., 1993).

During latency the genome is maintained in an episomal state and most of the genes are silenced except for one part of the long repeat that generates latency associated transcripts (LAT) (Stevens et al., 1987). The main ~8.5Kb LAT transcript is transcribed from the LAP1 promoter (Stevens et al., 1987) and then spliced into a 2Kb fragment (Farrell et al., 1991, Krummenacher et al., 1997). This is the most abundant form of LAT, present as an intron not capped or polyadenylated in a lariat structure (Coffin et al., 1998a, Farrell et al., 1991, Rodahl & Haarr, 1997, Wu et al., 1996, Zabolotny et al., 1997). In neurones, the 2Kb structure can be further spliced into ~1.5Kb fragment (Garber et al., 1997, Mador et al., 1998, Spivack et al., 1991).

It has not been possible so far to determine the exact function of the LAT transcripts but studies have shown this transcript to play a role in spontaneous reactivation (Leib et al., 1989, Perng et al., 1996). Also, studies using LAT

mutants have demonstrated that the absence of these transcripts seems to induce more apoptosis in trigeminal ganglia of both mice and rabbit upon switch from acute to latent infection (Ahmed et al., 2002; Perng et al., 2000).

Interestingly, latency seems to be maintained also by a coordinated immune response relying on CD8⁺T cells releasing IFN γ that prevents reactivation from sensory neurones (Liu et al., 2001a). In neurones, the latest model proposed is that after primary infection and a brief period of viral replication, macrophages and $\gamma\delta$ T cells initiate an infiltrate of CD4⁺T, CD8⁺T cells and macrophages that remain for the life time of infection. CD8⁺T cells position themselves in close contact with the neurone cell body whereas CD4⁺T cells localise to the axonal areas. The position of the CD8⁺T cells allows them to monitor for antigen presentation (gB in particular) on MHCI from the neurone in which case the CD8⁺T cell produces IFN γ or later in reactivation lytic granules that force the genome to remain in the latent state (Liu et al., 2001a).

Nevertheless, occasional reactivation of the latent virus is possible under conditions of stress or immunosuppression. In this case the CD8⁺T cell function is compromised and reactivation is induced with formation of nucleocapsid and glycoproteins for virion assembly (Khanna et al., 2004). These travel to the mucosal membrane where they can lytically infect the epithelial cells in the proximity of the infected neurones (secondary infection). Reactivation is not well understood yet mainly because of the difficulties in studying this process *ex vivo*, but it appears to happen in only very few neurones (Sawtell & Thompson, 2004).

1.3.4 HSV 1 as a gene delivery vector

HSV-1 can infect a wide range of cells including non dividing cells, cardiac cells (Coffin et al., 1996), dendritic cells and haematopoietic cells (Coffin et al., 1998b) as well as T and B cells from a culture of PBMCs (Papageorgiou et al., 2002), thus potentially allowing gene delivery for a wide range of applications including immunotherapy. Despite the large genome, many genes including toxic and replication-associated genes can be deleted without significantly affecting the rate of viral growth, enabling the introduction of up to 30Kb of exogenous DNA. Thus multiple full-length genes can be inserted. Genes encoding anti-inflammatory molecules or cytokines can also be included in addition to the immunogen, to favour or shift the immune response in a particular direction (Th1 vhs Th2). In addition, HSV1 has the advantage of having a relatively stable genome (Sarisky et al., 2000), and large stocks of high purity virus can relatively easily be grown. Moreover, life long latent infections can be established such that long term gene expression is possible. The fact that HSV-1 persists as an episome in the nucleus avoiding insertional mutagenesis is another advantage.

To be used as human gene therapy vector, the cytotoxicity of HSV-1 needs to be minimised. HSV-1 has been modified in numerous ways for use as a vaccine vector. This ranges from the simple inactivation or deletion of genes, to the use of replication incompetent viruses, the construction of HSV-1 based amplicons, and disabled infectious single cycle (DISC) viruses (Ali et al., 2002, Palmer et al., 2000, Willis et al., 2001). DISC are variants whose genomes do not contain the gene encoding the gH protein essential for infection thus allowing only a single cycle of infection of the virus.

Non-replicative HSV-1 can be obtained by deleting immediate early genes so as to block the viral gene expression cascade. Deletion of the essential ICP27 and ICP4 genes still allows the virus to replicate *in vitro* in complementing cell lines, while undergoing abortive replication *in vivo*. For complete ablation of cytotoxicity all IE genes should be deleted, requiring them to be provided *in trans* for virus growth. Unfortunately, the level of attenuation of the virus is inversely proportional to its ability to grow *in vitro*, making fully disabled viruses ideal for use in humans from a safety aspect, but impractical to produce (Ozuer et al.,

2002). Deletion of the VP16 gene would prevent the triggering of the lytic cascade avoiding having to delete all the IE genes. Unfortunately, although the activation domain of VP16 can be deleted (Smiley & Duncan, 1997), and efficiency of replication *in vitro* compensated for by the addition of HMBA or by infecting at high MOI (Yang et al., 2002), the VP16 coding gene can not be fully deleted, as VP16 is required for proper viral assembly and egress (Mossman et al., 2000b). Deletion of the whole gene prevents accumulation of enveloped cytoplasmic capsids although perinuclear enveloped virions can form but with altered morphology (Mossman et al., 2000b). Mutation at the C terminal activation domain of VP16 reduces or abolishes the transactivating function of the protein without affecting the structural integrity of the virus (Ace et al., 1989, Smiley & Duncan, 1997).

Safety may be further improved by deleting genes that are not essential for virus replication but are toxic, such as ICP22, or ICP34.5 (the major neurovirulence factor) (Chou et al., 1990), that can be deleted without significantly affecting the efficiency of virus production.

The choice of promoters is also important when using an HSV based vector since many have been found to be transcriptionally silenced at the onset of latency unless LAT promoters (which usually drive the expression of the only latency associate transcripts) which are the only HSV RNA transcripts produced in latency) are used to ensure long-term expression during latency (Lachmann & Efsthathiou, 1997, Lokensgard et al., 1997). Additionally, in neurones, the LAP1 and LAP2 promoter regions also allow long term expression from exogenous neighbouring promoters (Lewis & Emerman, 1994, Lilley et al., 2001, Palmer et al., 2000).

HSV 1 vectors have mainly seen application for gene delivery to the nervous system (CNS) (Lilley et al., 2001, Palmer et al., 2000, Perez et al., 2004), or in cancer therapy delivering cytokines (Liu et al., 2003; Sundaresan et al., 2000). However, HSV-1 also holds good potentials as an immunotherapy vector targeting DCs (Coffin et al., 1998, Samady et al., 2003; Willis et al., 2001). HSV-1 in an immune context has so far been used to stimulate response against the

virus itself (Mueller et al., 2002), against bacterial (Lauterbach et al., 2004), and viral infections (Murphy et al., 2000), or in cancer therapy (Liu et al., 2003).

1.3.4.1 Gene delivery to the CNS:

Because of its ability to naturally infect neurones, HSV1 seems an ideal gene delivery vector for the nervous system. Since more widely used promoters like CMV are silenced during latency, long term expression requires the use of LAT promoters (Lachmann & Efstathiou, 1997, Palmer et al., 2000).

Both replication competent (ICP34.5⁻ and inactivated VP16) and replication incompetent HSV vectors (ICP27⁻, and ICP4⁻) delivered via the sciatic nerve or footpad injection into mice have been shown to be transported and to express genes in the long-term in the dorsal root ganglia (DRG) (Palmer et al., 2000). Also, replication incompetent vectors have been shown to express marker genes in the central nervous system following injection into the brain or in the spinal cord of rats (Lilley et al., 2001). Finally, Perez et al (Perez et al., 2004) have recently shown that peripheral administration of attenuated vectors can efficiently mediate transport of genes to motoneurons of adult mice and rats, and long term expression can be achieved when using replication incompetent viruses.

1.3.4.2 Gene delivery for cancer therapy:

HSV-1 can be used as an oncolytic vector, with the ability to infect numerous cancerous cell lines *in vitro* (Liu et al., 2003) and replicate selectively in them. Relying on this mechanism, viruses deleted for the ICP34.5 gene could clear cancer cells both *in vitro*, in mice (Kesari et al., 1995, Randazzo et al., 1997), and subsequently in Phase I clinical trials (MacKie et al., 2001). Similarly, a virus deleted for ICP34.5 and ICP6 could treat malignant glioma in mice (Sundaresan et al., 2000), non human primates (Hunter et al., 1999), and has been tested in patients administered by intratumoral injection (Markert et al., 2000). Because of the ability of this virus to infect cancer cells but not bone marrow progenitor cells, it has also been used to “purge” cancerous cells from bone marrow cells *ex vivo*, although this has not yet been tested in patients (Wu et al., 2001).

Following these results, a vector based on a clinical strain of HSV1, has shown further improvement by deletion of the ICP47 gene in addition to ICP34.5, and also by including expression of GMCSF (Liu et al., 2003). So far this improved vector has demonstrated efficiency *in vitro*, and in mice where virus injection could clear the tumour and also protect against subsequent challenge (Liu et al., 2003), and is currently undergoing clinical trials.

1.3.5 HSV-1 for immunotherapy

1.3.5.1 Anti-HSV1 immune responses

In the development of a viral vector for immunotherapy, although a low level of protective immune response to the virus is potentially beneficial also to act as an adjuvant, a concern is that the antivector immune responses, either primary or pre-existing, might prevent efficient stimulation of the targeted response against the recombinant antigen. HSV can be modified to minimise toxicity therefore allowing the virus to persist for the expression of exogenous genes. However, viruses deleted for the immediate early genes are still immunogenic in vaccination studies in animals (Da Costa et al., 1999b). In addition, given the high proportion of already infected individuals, recall responses may cause a problem. This has, however, been addressed in studies in mice that have demonstrated that previous exposure to the virus does not appear to impede the establishment of a durable immune response from the incoming vaccine virus (Brockman & Knipe, 2002, Chahlavi et al., 1999), although in some cases recombinant gene expression has been shown to be reduced.(Herrlinger et al., 1998).

Both antibodies, complement (Verschoor et al., 2003), NK (Pereira et al., 2001), and NK cells (Grubor-Bauk et al., 2003) have been shown to be involved in the control of HSV infections *in vivo* in a Th1 type response (Brenner et al., 1994, Carr et al., 2001). HSV-1 infection results in an initial antiviral innate response followed by adaptive and acquired responses. The initial innate immunity mediates lysis of virally infected cells as well as providing a link to effective adaptive immunity (Wakimoto et al., 2003). When lesions occur there is an initial infiltration of NK and CD4⁺T cells and following CD4⁺T cell proliferative activity, CD8⁺T cells clear the infection (Koelle et al., 1998b)..

Complement plays a major role in fighting HSV-1 infection. Depletion of complement from serum can reverse the antiviral activity of the serum (Ikeda et al., 2000). The complement cascade involves a series of proteins that are activated by proteolytic cleavage leading to the formation of a membrane attack complex (MAC). This can lyse microorganisms and infected cells. These proteins can also recruit and allow opsonization by macrophages. The complement cascade can be initiated by two different signals. The classical pathway is initiated by an IgM molecule that recognises an antigen, or several IgG molecules binding to an antigen. The “alternative” pathway or the “lectin” pathway is triggered by mannose-binding proteins that bind sugar moieties present on the virus surface.

Antibodies binding to virus or virus infected cells have been shown to inhibit further HSV-1 infection by neutralisation and cell lysis via antibody-dependent cellular cytotoxicity (Dubin et al., 1992), opsonisation, and classical pathway of complement activation (Da Costa et al., 1999a). Both humans and mice that have been immunised or infected with HSV1 develop high titres of circulating antibodies, primarily IgG (Brenner et al., 1994, Carr et al., 2001, Peek et al., 2002). Naturally infected individuals usually develop antibodies against the viral envelope-bound glycoproteins, in particular gB, gC, gD, and over 90% of human adults possess circulating antibodies against this virus (Corey & Spear, 1986). Because these can bind viral particles, they have the potential to inhibit viral adhesion and entry, and to induce antibody-dependent cell mediated cytotoxicity. Interestingly, antibody immunity does not correlate with virus neutralisation *in vitro*, but correlates with the immune cytolysis in the presence of complement (Bystricka et al., 1997).

The humoral response to HSV is dependent on an intact classical complement pathway. Its effect is mediated via complement receptors CD21/CD35 (Da Costa et al., 1999a), and the C3 and C4 complement components produced by bone marrow derived myeloid cells. These C3 and C4 factors are critical and sufficient to induce B cell responses to inert proteins or infectious virus in response to a peripheral infection, implying that serum complement is probably not enough (Fischer et al., 1998, Gadjeva et al., 2002, Verschoor et al., 2003).

Whereas antibodies are expected to prevent the spreading of the virus, cellular responses are triggered to resolve peripheral lesions (Mikloska et al., 1999). Strong CD4 responses are present in HSV infected individuals, which seem to be the predominant cells secreting IFN γ in response to HSV (Asanuma et al., 2000), and depletion of CD4⁺T cells in mice result in increased susceptibility to challenge (Manickan & Rouse, 1995). In mice, immunisation with CD4 epitopes from gD can induce protective immune response to ocular challenge with HSV-1 (BenMohamed et al., 2003), and in humans, CD4 responses have been found targeted at proteins from the various stages of lytic infection, as well as targeted at the envelope, tegument and capsid proteins, and against intracellular proteins such as ICP8 (Koelle & Corey, 2003). In addition, numerous CD4 epitopes on VP16 and VP22 have been identified (Koelle et al., 1998a, Koelle et al., 2000, Kwok et al., 2001).

CD8⁺T cells follow the infiltration of CD4⁺T cells in the lesions (Cunningham et al., 1985), and infiltration of CD8T cells, but not that of CD4 or NK cells, correlates with clearance of infection (Kurtzhals et al., 1998, Watanabe et al., 1994). CD8⁺T cells can clear infection from peripheral nerve endings as well as from the skin (van Lint et al., 2004), and may also play a role against intraocular infection (Watanabe et al., 1994).

Depletion of CD8⁺T cells in Balb/c initially failed to completely clear the virus from sensory ganglia, associating clearance of the lytic infection in the ganglia with CD8⁺T cells (Simmons & Tscharke, 1992, Speck & Simmons, 1998). This response does not involve killing of neurones, instead, replication is terminated in a cytokine, IFN γ dependent way (Liu et al., 2001a, Liu et al., 2000a, Martz & Gamble, 1992, Pereira et al., 2000, Speck & Simmons, 1998).

The IFN γ released seems to inhibit ICP0 production during the lytic cycle by decreasing the amount of cdk2 and cdk4 available (required for viral reactivation, replication and for post-translational modifications to activate ICP0). In addition, IFN γ strongly upregulates expression of the various components of the ND10 body to prevent transcription (Khanna et al., 2004)

Both humans and mice HSV-1 infections result in increased NK cell activity (Fitzgerald et al., 1985, Habu et al., 1984). These cells are the first line of protection against viral infections and can kill malignant and virus infected cells without antigen presensitisation. Their main function is to release IFN γ and they have also been shown to also be important in controlling reactivation of the virus (Biron, 1997, Bukowski et al., 1983). In their absence, individuals are highly susceptible to progressive HSV infections (Biron et al., 1989). $\gamma\delta$ T cells are also activated upon HSV-1 infection with the role of producing IFN γ for macrophage stimulation that help in restricting the growth of HSV-1 (Cheng et al., 2000). Finally depletion studies in mice have shown that neutrophils also contribute to limiting the spread of viral infection in the eye, trigeminal ganglia and brain (Tumpey et al., 1996), providing initial clearance of the virus and also helping in CD4⁺T cell function (Thomas et al., 1997).

There is a persistent high level of cytokines during latency, implying that cytokines play an important role in the maintenance of latent infection to prevent reactivation. Type I IFNs have the most powerful antiviral property. These can render cells relatively non-permissive for productive infection (Leib et al., 1999). Looking at the effect of infection in mice deficient for IFN α/β signalling pathways, it emerged that these cytokines limit HSV-1 infection and spread (Hendricks et al., 1991, Leib, 2002, Leib et al., 1999), whereas *in vitro* experiments showed interference of IFN α/β with IE gene expression, (Mittnacht et al., 1988, Oberman & Panet, 1988) presumably by interacting with the VP16 initiation complex (De Stasio & Taylor, 1990).

Type I IFN initiates innate and specific immune responses (Riffault et al., 1996) and also acts directly to inhibit viral replication (Leib et al., 1999). IFN α/β do not induce an antiviral response on their own, but they activate a number of interferon-stimulated genes (ISG), that can also be stimulated directly by viral infections, and together function to inhibit viral replication and spread (Collins et al., 2004). The complementary viral RNA that forms dsRNA can activate the cytoplasmic protein IRF3 (IFN regulatory factor 3) that in turn translocates to the

nucleus to activate the IFN β gene. This is then secreted and binds to the receptors of the same cells as well as to the neighbouring cells leading to activation of the IFN signalling pathway. Through the action of kinase (eg Jak1) and proteins (eg Stat2), this leads to the formation of an IFN stimulated growth factor 3 complex (ISGF3) that activates IFN stimulated response elements (IREs). Viral replication is then inhibited by an oligo-adenylate synthase (OAS) that activates RNaseL that in turn inhibits viral replication and activation of dsRNA-dependent protein kinase (PKR) that usually allows translation and thus viral replication.

As one of the most effective host antiviral responses, the virus has developed numerous mechanisms to overcome it, such as expression of ICP34.5 to target PKR (discussed later) or ICP0, which blocks ISG induction through a proteasome dependent mechanism not yet elucidated (Eidson et al., 2002).

The other major cytokine released following infection is TNF α , produced by activated macrophages. Like Type I IFNs, TNF α can inhibit viral infection and replication (Kodukula et al., 1999). Enhanced reactivation and more frequent primary acute corneal infection can be seen in trigeminal ganglia explants of TNF α knockout mice implying that this cytokine plays an important role both during primary responses and during latency to avoid reactivation (Minagawa et al., 2004).

1.3.5.2 HSV-1 Immune evasion mechanisms

The efficiency of a vector for immunotherapy relies on the adequate stimulation of the immune system, usually obtained by appropriate stimulation of DC. Although it is important for the efficiency of gene delivery to understand but minimise the host response to the virus, in the construction of a vector targeted at stimulating an immune response, particular attention needs to be drawn to the mechanisms used by the virus to silence the numerous antiviral responses.

Herpesviruses all express genes that aid evasion of the immune response. Genes coding for proteins that cause false positive cell signalling such as the G protein coupled receptor, bcl2 anti-apoptotic protein analogue, and viral cyclin are

common to most γ herpesviruses. HSV-1 expresses ICP47, ICP34.5 and vhs amongst others, that act to block host responses.

The ability to infect and modulate the activity of DCs is a powerful method by which the virus escapes immune responses. HSV-1 infected DCs are poor stimulators of T cell responses (Pollara et al., 2003, Salio et al., 1999). HSV1 can infect DCs efficiently (Coffin et al., 1998b). Up to 90% of dendritic cells can be transduced at MOI as low as 1-5 (Kruse et al., 2000b, Salio et al., 1999), because of the presence of HVEM and nectin receptors on the surface of the cell. HSV-1 preferentially infects immature cells (Salio et al., 1999) and although some replication has been reported from immature cells (Mikloska et al., 2001), the virus seems to infect DC non-productively, showing only IE and E transcripts production in mature DCs (Albers et al., 1989, Mikloska et al., 2001, Salio et al., 1999). Immature cells undergo apoptosis soon after infection (Mikloska et al., 2001) whereas mature cells can remain viable (Kruse et al., 2000b).

As an immune evasion mechanism, infection with wild type (Kruse et al., 2000b) or DISC (Salio et al., 1999) blocks cell maturation, and therefore antigen presentation. This is evident by the downregulation of expression of costimulatory markers (especially CD86) and antigen presentation molecules (both MHCI and MHCII) which is observed, as well as a decrease in the level of IL12 secretion upon stimulation of maturation, resulting in apoptotic cell death (Pollara et al., 2003). Interestingly, despite the productive infection in immature DC, costimulatory molecules still undergo downregulation (Mikloska et al., 2001). This shows that HSV-1 infects DC and interferes with their activity, both with regard to maturation, and to antigen presentation to T cells. These effects are probably a result of a coordinated activity of a number of viral genes.

Both MHCI and MHCII loading mechanisms can be disrupted by infection through the action of ICP47 and gB respectively. ICP47 acts on a transporter associated with antigen processing (TAP) by inhibiting peptide transport into the ER lumen. Experiments carried out in mice have shown that deletion of the ICP47 genes results in higher CD8⁺T responses and recognition of human DCs and

decreased neurovirulence in mice (Goldsmith et al., 1998, Tigges et al., 1996). Interestingly, the action of ICP47 has been shown not to be very effective in mouse DC (Ahn et al., 1996). On the other hand, interference with MHCII presentation seems to take place as a result of reduced expression of the invariant chain for correct MHCII peptide loading. gB competes for binding of the invariant chain, thus forming complexes with DM and DR molecules that are prevented from reaching cell surface (Neumann et al., 2003).

The vhs protein plays a major role in the inhibition of the functions of DCs. Evidence that vhs has an immune evasion role and should be inactivated to obtain a vector able to induce a response came from the observation that vhs null viruses are more efficient at stimulating protective responses against HSV infection (Geiss et al., 2000, Walker et al., 1998, Walker & Leib, 1998). The ability of this protein to interfere with mRNA synthesis by various mechanisms (Sydiskis & Roizman, 1966) as discussed earlier (§1.3.2.2) is a major evasion mechanism, that results in multiple effects. Mainly, vhs impairs the ability of DCs to stimulate T cells and this takes place by various means (Kruse et al., 2000b, Salio et al., 1999). Vhs acts in conjunction with ICP47 to downregulate expression of MHCI. Mutant viruses have shown that this protein is responsible for a decrease in MHCI presentation by decreasing the level of mRNA in the cell (Hinkley et al., 2000, Koppers-Lalic et al., 2001, Tigges et al., 1996). Similarly, MHCII presentation has also been shown to be impaired by the action of vhs (Trgovcich et al., 2002). Vhs is also involved in suppressing production of proinflammatory cytokines and chemokines including IL8, MIP1 α , IL1 β (Suzutani et al., 2000).

Vhs also plays a role in interfering with type I IFN responses. ICP0 and vhs seem to act together to inhibit the host antiviral IFN $\alpha\beta$ response. ICP0 appears to use its RING finger domain to block interferon regulatory factor 3 and 7 (IRF3, IRF7) mediated activation of IFN stimulated genes (Lin et al., 2004). ICP0 also seems to downmodulate the expression of IFN γ induced antiviral proteins by targeting them for destruction by proteasomes (Chee et al., 2003, Lin et al., 2004)

Treatment of cells infected with a vhs- HSV-1 virus with IFN produces reduced levels of late genes as compared to cells infected with wild type virus (Chee & Roizman, 2004). On the other hand, cells lacking the IFN $\alpha\beta$ receptor can sustain normal replication by vhs- HSV-2, that although this may replicate well *in vitro*, they replicate poorly *in vivo* and have decreased virulence (Duerst & Morrison, 2004). This mechanism has been shown to involve vhs interfering with the PKR response induced by dsRNA. In fact, enhanced replication is obtained in the absence of PKR. Vhs also possibly interferes with the role of RNaseL (Khabar et al., 2000, Murphy et al., 2003). Recently, in HSV-1 studies, vhs has been shown to be involved in interference with the IFN $\alpha\beta$ response possibly by downregulating Jak1 and Stat2. This would reduce IFN receptors on the cell surface (Jak1) and interfere with IFN signalling (Stat2). Together, these effects probably account for the reduced formation of complexes on interferon induced response elements (Chee & Roizman, 2004). Interestingly vhs from HSV1 had previously been shown to interfere only minimally with the response to IFN $\alpha\beta$ *in vitro* (Mossman et al., 2000a) and *in vivo* (Leib et al., 1999). HSV1 and HSV2 therefore seem to interact differently with IFN responses. Vhs from HSV2 has been suggested to be more effective than that from HSV1 and may act more efficiently to decrease the levels of IFN $\alpha\beta$ (induced by viral infection) and of ISG transcripts (induced by receptor stimulation) (Duerst & Morrison, 2004).

In addition to the reduced MHCI and MHCII presentation and the interference with the type I IFN responses, HSV-1 infection also induces a decrease in the expression of CD83 on the cell surface (Kruse et al., 2000b, Salio et al., 1999). CD83 is completely degraded in lysosomal compartments (Kobelt et al., 2003) within 10hr of infection by blockage of mRNA transport from the nucleus, therefore resulting in reduced stimulatory capacity of the infected cells (Kruse et al., 2000a, Kruse et al., 2000b).

The viral protein ICP34.5 and the protein encoded by the US11 gene also function against the antiviral response. In infected cells, PKR phosphorylates the α subunit of the eIF2 α translation initiation factor, therefore blocking translation of viral proteins. ICP34.5 encodes a regulatory subunit that activates a cellular protein

phosphatase (PP1) that dephosphorylates eIF2 α (He et al., 1997, He et al., 1998). Similarly, the dsRNA binding protein expressed by US11 can prevent eIF2 α phosphorylation by inhibiting to an eIF2 α kinase (Mohr, 2004).

Finally, glycoproteins expressed by the virus can interfere with the complement pathway. gC binds the C3 complement factor thus inhibiting its antiviral action (Lubinski et al., 1998), whereas gE and gI act by forming a complex that inhibits the classical complement cascade. IgG molecules can be produced and bind to viral gB, gC and gD (required for cell entry) via their Fab domain. Normally, this would trigger the classical complement pathway, leading to phagocytosis of the virus, but instead, the gE-gI complex can bind to the Fc domain of IgG (Dowler & Veltri, 1984) therefore blocking the immunoglobulin from activating the complement cascade.

Nonetheless, it has been suggested that despite downregulation and impairment of function of infected DCs, surrounding uninfected DCs are stimulated to mature by soluble factors released by the dying infected cells. This suggests that uninfected DCs can cross present antigens from infected DCs and undergoing maturation and possibly T cell activation (Pollara et al., 2003). Both Belz (Belz et al., 2004) and Allan (Allan et al., 2003) have shown that different types of DCs (those in the lung airway, or Langerhans cells in the skin epidermis) can be infected by HSV-1 but these may not be the cells involved in the presentation of antigen in the LN where a population of CD8 α^+ cells is associated with antigen presentation of MHCI. Similarly, footpad injection of HSV-1 resulted in gB antigen presentation in the LN by a CD8 α^+ DC subset (Smith et al., 2003). Taken together, these results imply that it is possible to target DCs with HSV-1 and achieve presentation in the LN, but the immune evasion mechanism acquired by this virus impair the function of DCs that therefore may present antigens via cross-presentation.

1.3.5.3 Uses of HSV-1 for immunotherapy

Potent immune responses can only be obtained by fully mature DC efficiently presenting the appropriate peptides and with a full set of costimulatory molecules. Therefore, to use HSV1 as a vector for immunotherapy, it is crucial to modify it so as to allow this level of cell maturation following infection. So far, because of the inhibiting properties of HSV-1 on dendritic cells, HSV-1 based amplicons (plasmid based vector packaged into an HSV-1 capsid) or replication incompetent viruses have been used to induce immune responses. Using an HSV-1 based amplicon expressing a tumour antigen it has been possible to show efficient transduction of dendritic cells and induction of antigen specific T cells after mouse injection of transduced DCs (Willis et al., 2001). In another study, *ex vivo* transduction of DC with an HSV-1 amplicon expressing marker genes could successfully express the genes and stimulate a reaction in MLR, but, interestingly, these cells *in vivo* could only stimulate antibody and no cellular responses to the genes (Nunez et al., 2004).

HSV has also been tested as a DISC. A DISC virus expressing mGMCSF showed a therapeutic effect inducing a strong CTL response against established murine colon carcinoma after either direct intratumoral injection of the vector, or, even better, after injection of infected DCs (Ali et al., 2002). Importantly, this study also showed that previous exposure to the virus did not affect the efficiency of vaccination.

Overall, taking into account the known function of specific HSV-1 genes that interfere with the immune system it is possible to construct vectors that should allow maximal expression of recombinant genes upon infection of DCs. Recombinant replication incompetent HSV have mainly been used to induce responses against viral antigens, mostly HSV (Da Costa et al., 1999b, McLean et al., 1994, Morrison & Knipe, 1996), but attenuated vectors have also proved capable of stimulating a response in rhesus monkeys against simian immunodeficiency virus (Murphy et al., 2000). Recently, a replication incompetent HSV-1 (ICP4⁻, ICP22⁻, ICP27⁻, vhs⁻) expressing the test antigen ovalbumin has also been used showing efficient stimulation of protective CD8⁺CTL responses against intracellular bacteria expressing the same antigen. A

single immunisation dose could stimulate long term protective CD8⁺ responses while stimulating very low amounts of CD4⁺T cells and antibodies, with an efficiency stronger than DNA gene gun immunisation (Lauterbach et al., 2004), showing the advantage of using a viral vector and the potentials of using HSV-1 viruses for immunotherapy purposes.

1.4 Hypothesis and Aims of this thesis:

The hypothesis behind this work is that HSV-1 can be modified and attenuated so as to allow it to infect dendritic cells without preventing them from undergoing maturation. It should then be possible to use this viral backbone to express foreign antigens such as malaria antigens, and use this recombinant virus to induce both IFN γ release and antibody responses to the recombinant proteins and to the virus *in vivo*.

The aim of this thesis is to construct an HSV-1 based immunotherapeutic vector able to stimulate immune responses to malaria. The virus has to be able to transduce DCs efficiently, and allow them to undergo unaltered maturation, and trafficking to the lymph node so as to stimulate a maximal immune response to the delivered antigen, possibly by additional expression of immunostimulatory cytokines. Moreover, the vector should express multiple full-length genes and be able to stimulate both cellular and humoral responses to them. Finally, the constructed recombinant virus should stimulate an immune response both upon injection of ex-vivo transduced dendritic cells and upon direct injection of neat virus.

Chapter 2

Materials and Methods

2.1 General reagents and suppliers

2.1.1 General Suppliers

General disposable plastic ware:

Greiner Ltd. (Stonehouse, Gloucester, UK).

Sterilin Ltd (Stone, Staffordshire, UK)

General analytical grade chemicals:

Merck Ltd (Poole, Dorset, UK)

Boehringer Mannheim (Lewes, East Sussex, UK)

Sigma chemical Company Ltd (Poole, Dorset, UK)

Tissue culture media, solutions and supplements for fibroblast and primary cultures:

Gibco-BRL Life Technologies Ltd., Renfrewshire, Scotland, UK.

Tissue culture plastic ware:

Nunc (Roskilde, Denmark)

3MM chromatography paper:

Whatman International Ltd (Maidstone, Kent, UK)

Disposable filters 0.45µm and 0.2µm:

Gelman Life Sciences (Ann arbor, Michigan, USA)

Kodak X-OMAT imaging photographic film:

Sigma Chemical Company Ltd (Poole, Dorset, UK)

Restriction and modifying enzymes and buffers:

Promega Corporation (Madison, Wisconsin, USA)

Antibodies

DakoLtd. (High Wycombe, Bucks, UK)

Serotec

Insight Biotechnology Ltd.

2.1.2 Plasmids

pGemTeasy vector system I: **Promega Corporation** (Madison, Wisconsin, USA) was used according to manufacture instructions for the cloning of PyCS and PySSP2 genes

pBluescript: **Stratagene Ltd**, Cambridge, UK

pcDNA3: **Invitrogen Corporation**, Carlsbad, California, USA

phrGFP-1: **Stratagene Ltd**, Cambridge, UK

pDsRed1-N1: **Clontech**, BD Biosciences, Franklin Lakes, NJ, USA

pCMVPyCS : This plasmid was obtained from Dr M.Tsuji (NewYork University School of Medicine, NY). It contains the full length sequence of PyCS (amino acids 1-356) without the terminal putative GPI-anchor sequence.

pBs43NP: This plasmid was present in the laboratory and expresses the full length NP gene from H₃N₂ strain of influenza.

2.1.3 Genomic DNA

The genomic DNA of *P. yoelii* 17XNL (www.tigr.org) was a gift from Prof.A.Holder, (NIMR, London, UK)

2.1.4 Bacterial strains

The Escherichia Coli XL-1 Blue; this strain was used to carry out the cloning for all plasmids in this project.

Genotype: *recA1 end A1 gyr196 thi-1 hsdR17 supE44 relA1 lac [F'proAB LacI^r ZΔM15, Tn10(Tet)^r]*

Source: **Stratagene Ltd**, Cambridge, UK

2.1.5 Antibodies

Antibody	Application-Dilution	Detection	Dilution
Hamster anti-CD11c ⁺ biotin conjugated (<i>BD Pharmingen</i>)	FACS 1:50	Streptavidin-Red670 (<i>Gibco</i>)	1:100
Rat anti-mouse CD40 (<i>Serotec</i>)	FACS 1:50	Goat anti-rat PE conjugated (<i>Serotec</i>)	1:50
Rat anti-mouse CD80 (<i>Serotec</i>)	FACS 1:50	Goat anti-rat PE conjugated (<i>Serotec</i>)	1:50
Rat anti-mouse CD86 (<i>Serotec</i>)	FACS 1:50	Goat anti-rat PE conjugated	1:50

		(Serotec)	
Rabbit anti-HSV1 (DAKO)	FACS 1:50	Sheep anti-rabbit PE conjugated (Serotec)	1:50
Rabbit anti-HSV1 HRP conjugated (DAKO)	Immunostaining 1:100	Swine anti-rabbit HRP conjugated (DAKO)	1:100
Mouse anti-human CD40 (Insight)	FACS 1:50	Rabbit anti-mouse biotin conjugated (Serotec) Streptavidin-Red 670 (Gibco)	1:100 1:250
Mouse anti-human CD86 (Insight)	FACS 1:50	Rabbit anti-mouse biotin conjugated (Serotec) Streptavidin-Red 670 (Gibco)	1:100 1:250
Mouse anti-human CD80 PE conjugated (BD)	FACS 1:50		
Mouse anti-human CD19 (Insight Biotechnology)	Depletion 0.5µg/10 ⁶ cells		
Mouse anti human CD3 (Insight Biotechnology)	Depletion 1µg/10 ⁶ cells		
Mouse anti-human CD2 (Insight Biotechnology)	Depletion 0.5µg/10 ⁶ cells		
Mouse anti-influenza A Nucleoprotein (NP) (Serotec)	Western blot 1:200 ELISA 1:200	Rabbit anti-mouse HRP conjugated (DAKO)	1:1000
Anti-PyCS (9D3) ascite (Dr M.Tsuji New York University School of Medicine, NY)	Western blot 1:300 ELISA 1:300	Rabbit anti-mouse HRP conjugated (DAKO)	1:1000

Anti-PySSP2 hybridoma Supernatant <i>(Dr A.Rodriguez Dep.Medical and Molecular Parasitology New York University, NY)</i>	Western blot 1:2 ELISA 1:2	Rabbit anti-mouse HRP conjugated <i>(DAKO)</i>	1:1000
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Table 2.1: Antibodies used in this thesis together with the dilution used for each application and the detection antibody used.

2.1.6 Standard solutions and buffers

Luria Bertani media (LB): 1% (w/v) Bacto®-tryptone, 1% (w/v) NaCl, 0.5 % (w/v) Bacto®-yeast extract. This was then autoclaved at 120°C 20 minutes.

Phosphate buffer solution (PBS): 137mM NaCl, 2.7mM KCl, 4.3mM disodium protophosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$), 1.4mM potassium hydrogen orthophosphate (KH_2PO_4), pH7

TAE: 400mM Tris base, 200mM sodium acetate, 20mM EDTA pH8.3

SSC: 150mM NaCl, 15mM sodium citrate

2.2 Molecular Biology

2.2.1 Propagation of bacteria

Liquid cultures of XL1-Blue were grown in Luria Bertani media (LB) media containing either no antibiotic, 100µg/ml ampicillin (Sigma), or tetracycline at a final concentration of 10µg/ml. Cultures were placed overnight in an orbital shaker (New Brunswick Sci Series 25) at 200rpm at 37°C. Stocks of ampicillin (Sigma) were made at 100mg/ml (1000X) and stored at -20°C. Colonies of XL-1 Blue were isolated by growing on LB plates containing 2% Bacto®-agar and ampicillin at 100µg/ml or tetracyclin at 12.5µg/ml.

For long term storage of bacteria an overnight culture was diluted 1:100 into fresh LB media and incubated shaking at 200rpm 37°C for an hour after which an equal volume of 100% glycerol was added and the two solutions thoroughly mixed. The glycerol/bacteria solution was aliquoted into cyto-vials and frozen at -80°C.

2.2.2 Production of competent bacteria

This method of preparation of competent XL-1 Blue cells and transformation is based on the calcium chloride method described by Sambrook (Sambrook et al 1989). A single bacteria colony was grown overnight in 10ml of LB without antibiotic. This culture was then diluted 1:100 in LB without antibiotic selection and grown for approximately 2hr until reaching OD₅₈₀ of about 0.5 units. The cells were then pelleted in 50ml sterile tubes by centrifugation at 1496g (3200rpm) for 10minutes at room temperature. Supernatant was discarded and the cells resuspended in 10ml of ice cold 100mM calcium chloride (CaCl₂) and left on ice for 2hr. The bacteria were then pelleted again by centrifugation at 1496g for 10min at 4°C, the supernatant discarded and cells resuspended in 2ml ice cold 100mM CaCl₂. These competent cells were kept on ice and used within 48hr.

2.2.3 Transformation of bacteria

Competent cells were transformed by addition of DNA to 1ml of cell and incubation on ice for 30min. These were then heat-shocked at 42°C for 90seconds and placed immediately on ice for a further 5minutes after which 4ml of LB without antibiotic selection were added and the mixture was placed in the orbital

shaker at 200rpm 37°C for 1hr. The cells were pelleted by centrifugation at 1496g (3200rpm) for 10min at room temperature, the supernatant decanted and the pellet resuspended in 100µl of LB. Cells were plated onto LB plates containing the appropriate antibiotic selection and these placed in an incubator 37°C overnight.

2.2.4 Small scale plasmid DNA extraction (mini-prep) from transformed bacteria

This “mini-prep” method of DNA extraction is based on the alkaline lysis method previously described by Birnboim (Birnboim & Doly, 1979). Individual colonies of transformed XL-1 Blue cells were used to inoculate 3ml of LB containing the appropriate antibiotic selection and placed overnight in the orbital shaker (200rpm, 37°C). 1.5ml of culture were then pelleted by centrifugation in a bench top centrifuge at 425g (2000rpm) for 2minutes, the supernatant was then removed by aspiration and the pellet resuspended in 100µl of resuspension buffer (50mM Tris-HCl pH 7.5, 10mM EDTA pH8, 100µg/ml RNase-A). The cells were then lysed by addition of 200µl of lysis buffer (200mM NaOH, 1% (v/v) Triton X-100) and neutralised by addition of 150µl of neutralisation buffer (3M KOAc pH4.8). The cell lysate was vortexed and then centrifuged for 5minutes at 425g (2000rpm) and the pelleted precipitate was removed with a hypodermic needle bent at the tip, and discarded. 500µl of isopropanol were then added to the supernatant that was vortexed and centrifuged for 5minutes at 425g. After removal of the supernatant, the DNA pellet was washed with 500µl of 70% ethanol (EtOH) and the DNA pellet was then dried under vacuum and resuspended in 50µl of double-distilled water (ddH₂O). Plasmid DNA was stored at -20°C.

2.2.5 Large scale plasmid DNA extraction from transformed bacteria

For extraction and precipitation of large scale plasmid DNA, a single transformed colony (or 100µl of culture) was used to inoculate 400ml of LB containing the appropriate antibiotic selection. This was placed in an orbital shaker (200rpm, 37°C) overnight. 100ml of this culture were centrifuges at 1710g (3000rpm) for 10minutes and the supernatant discarded. Plasmid DNA was then extracted using the Marligen Midi-Prep Kit (*Biosciences Inc*) according to manufacturers

instructions. The purified DNA was dried and resuspended in 100µl of ddH₂O and the average yield using this method was 100µg.

2.2.6 Restriction enzyme digestions

Restriction digests were performed on plasmid DNA either for analysis or for isolation of DNA fragments. Analytical digests were performed in a total volume of 20µl containing 5µl of mini-prep DNA or 1µl of midi-prep DNA. 10 units of enzyme were added together with the appropriate buffer as specified by the manufacturer. Digests were incubated 1-2 hours at the appropriate temperature. The digests were then run on a 1% agarose gel and the bands visualised on a UV transilluminator.

Restriction digests required for the isolation of DNA fragments were carried out in a total volume of 40µl containing 2-3µg of midi-prep DNA and the appropriate buffer. Digests were incubated 2-16 hours at the appropriate temperature and then run on a 1% agarose gel. DNA bands were visualised on a UV transilluminator and the band corresponding to the required fragment carefully excised using a scalpel. The DNA was then extracted from the agarose using the GFX PCR DNA and Gel Band Purification Kit (*Amersham Biosciences*) following the instructions of the manufacturer. DNA was then eluted in a final volume of 50µl.

2.2.7 Blunting treatment

If compatible ends were not available to perform ligation of two fragments the digested DNA was purified and then the 3' end overhangs were blunted using T4DNA polymerase (Promega). 1µl of 25mM stock of dNTP (dATP; dCTP, dGTP, dTTP) and 15units of T4DNA polymerase were added to the DNA and incubated at 37°C for 20minutes. This reaction was then inactivated by heating at 90°C for 5 minutes and cooled on ice before proceeding with additional manipulations.

2.2.8 Phosphatase treatment

When digesting DNA vector plasmids with enzymes that leave compatible or blunt ends, these were treated with calf intestinal alkaline phosphatase (CIAP) (*Promega*) to avoid religation of the two ends. The reaction was carried out in a

total volume of 100µl using 10 Units of CIAP with the appropriate buffer and the reaction was left at 37°C for 30 minutes. The DNA was then purified using the GFX PCR DNA and Gel Band Purification Kit (*Amersham Biosciences*).

2.2.9 Agarose gel electrophoresis

1% (w/v) agarose (*Invitrogen*) gels were made using 1 x TAE (0.4M Tris, 0.2M sodium acetate, 20mM EDTA, pH8.3). Ethidium bromide was added to a final concentration of 0.5µg/ml. The gel was poured into a gel caster and left to set with a comb. 1Kb plus DNA ladder (*Invitrogen*) was used as size marker and DNA samples mixed with 1 x loading buffer (1 x TAE, 50% v/v glycerol, 0.025% bromophenol blue) were loaded on the gel that was usually run at 60V to allow good separation of the bands. Bands were then visualised on a UV transilluminator.

2.2.10 Ligation reaction of DNA

1µl of purified vector and 1µl of purified insert were run on a 1% agarose gel to empirically determine the amount present. Ligations were carried out in a total volume of 20µl, containing 1-2µl of gel purified vector, 7-10µl of gel purified insert depending on the initial concentration of the fragments but usually obtaining a 1:3 vector:insert proportion. 1 x ligase buffer was added to the mix together with 1-3 units of T4 DNA ligase (*Promega*) in ddH₂O. Reactions were carried out at room temperature for 2 hours and the enzyme then inactivated by incubation at 80°C 5 minutes. The ligation reaction was then transformed into 1ml competent XL-1 blue cells.

2.2.11 Polymerase chain reaction (PCR)

The specific PCR amplification of a target DNA sequence from *P. yoelii* genomic DNA was achieved using methodology essentially as described by Saiki et al (1985) and in *www.tigr.org*. Conditions used for the specific antigens are shown below. A working stock of *P. yoelii* 17XNL genomic DNA 7µg/ml (1:100 dilution of initial stock) was prepared in autoclaved ddH₂O. Samples for the PCR were prepared in a total volume of 50µl, using 5µl of DNA, 1µl of 10µM forward and reverse primers, 1µl of 10µM dNTP mix (dATP, dCTP; dGTP, dTTP), 1 x pfu buffer and brought to volume with autoclaved ddH₂O. The pfu enzyme was added

after the heat start as 2µl of pfu mix (5µl pfu, 2µl pfu buffer, in total 20µl). Pfu was the preferred enzyme used because of its proofreading activity. A negative control sample with ddH₂O replacing DNA was included in each run to check for contamination.

To initially determine the ideal annealing temperature the samples were run in a thermocycler (*Eppendorf Master Cycler Gradient*) using a gradient of temperatures varying between 45-65°C (45°C, 50°C, 55°C, 60°C, 65°C). To obtain enough DNA to carry out the cloning, the fragments were amplified twice. 5µl of the PCR product were run on a 1% agarose gel to ensure correct amplification. The rest of the sample was then purified using a GFX PCR DNA and Gel Band Purification Kit (*Amersham Biosciences*) and resuspending in 25µl of ddH₂O, the gene was then inserted into the pGemTeasy vector (*Promega*) as specified by manufacturer.

Primer name	Primer sequence	Location
PySSP FW	5'-GAGGGATCCGAAAAGACAGAAACA TGAAGCTCTTA-3'	ATG of SSP2 gene
PySSP RV	5'-GAGGGGATCCAAAATTAGTTCCAGT CATTATCTTC-3'	Stop codon bp2996
NP int FW	5'-GAAAGTGGATGAGGGAAC TCG-3'	bp350
NP int RV	5'-CCTCTCCAGAAGTTCCGATCAT-3'	bp671
T3	5'- ATT AAC CCT CAC TAA AG-3'	Sequencing primer
T7	5'- GAT ATC ACT CAG CAT AA-3'	Sequencing primer

Table 2.2: Primers sequences used to PCR amplification and their location in the gene

PCR cycles

Py SSP2 amplification: This is a ~2.5Kb gene therefore requiring a 5 minutes elongation time. The ideal annealing temperature for this set of primers was 60°C. The heat start cycle used was therefore: 94°C 6 minutes, 94°C 1 minute, annealing at 55°C for 2 minutes, and synthesis at 72°C for 5 minutes repeated a total of 35 times.

Amplification using NP internal primers: These primers were used with the cycle: 95°C 1min, 95°C 30sec, 45°C 30sec, 72°C 2min repeated 30 times.

The amplified samples were then run on a 1% agarose gel.

2.2.12 Reverse transcription PCR (RT PCR)

All solutions used for this procedure were made using diethylpyrocarbonate (DEPC) treated water.

2.2.12.1 Isolation of RNA

RNA was extracted from 5×10^6 infected cells using Tri reagent (*Sigma*). Cells were incubated with 1ml of Tri reagent for 5 minutes at room temperature after which 0.2ml of chloroform was added and the tube shaken vigorously for 15sec. The mix was then incubated at room temperature for 10minutes before being centrifuged at 12,000g for 15mins at 4°C. Following centrifugation the aqueous phase containing the RNA was transferred to a fresh tube and mixed with 0.5ml isopropanol, stored at room temperature 5-10 minutes and centrifuged at 12,000g for 8mins at 4°C to allow RNA precipitation. The pellet was washed with 75% ethanol and then air dried and resuspended in 20µl of DEPC treated H₂O.

2.2.12.2 Synthesis of cDNA

The RNA was then reverse transcribed using the OneStep RT-PCR kit (*Quiagen*) as described by the manufacturer and using the PCR cycle optimised for internal primers (§ 2.2.11). Internal primers were used because of the possibility of RNA degradation. As negative control, to ensure absence of genomic DNA contamination in the RNA samples, these were amplified using the same cycle but in a normal PCR reaction mix.

2.2.13 Quantification of DNA

Following plasmid DNA extraction this was quantified with a spectrophotometer. 5µl of DNA sample were diluted 200 fold into 1ml of ddH₂O and the absorbance was read to A₂₆₀. Absorbance of 1 at A₂₆₀ corresponds to 50µg/ml, the reading was therefore multiplied by the dilution factor (x200) and by the conversion factor (x50) before being divided by a factor of 10³ to obtain a measurement of DNA as µg/ml.

$$[\text{DNA}] = (\text{A}_{260} \times 200 \times 50) / 1000 \mu\text{g ml}^{-1}$$

2.2.14 DNA sequencing

Plasmid DNA was purified using phenol/chloroform. DNA at a concentration of 50ng/μl was sequenced by Department of Biochemistry, University of Cambridge, Cambridge, UK. The primers for each case were used at a concentration of 10pmol/μl.

2.2.15 Analysis of DNA by Southern blot

2.2.15.1 Southern blot

Southern blots (Southern, 1975) were performed on viral DNA to confirm the genome structures of newly constructed recombinant HSV-1 vectors. 10-30μl (depending on the density of the DNA preparation) of viral DNA were digested overnight with the appropriate enzyme and buffer in a total volume of 50μl. The enzyme was chosen in such way as to cut at both ends of the recombinant protein to obtain a band corresponding to the protein that could be easily identified. Viral DNA from a virus not containing the recombinant protein was also digested as a negative control, and 0.1μg of plasmid DNA used to construct the recombinant virus was digested in a total volume of 20μl to be used as a positive control. The digestions were then loaded and run on a 1% agarose gel until bands were well separated.

The DNA was then visualised on a UV transilluminator and photographed against a fluorescent ruler. The gel was then left on the transilluminator for 2 minutes to degrade the DNA and was then placed into denaturing solution (1.5M NaCl, 0.5M NaOH) on a platform gently shaking at room temperature for exactly 45 minutes. The gel was then rinsed with water before being neutralised with neutralisation solution (1M Tris pH5.5, 2M NaCl) for at least 30 minutes on a shaking platform. The transfer was then assembled by placing the gel upside-down on a plastic support previously layered with 3MM chromatography Whatman paper (*Whatman International Ltd*) absorbing solution from a recipient containing 20 x SSC (1 x SSC: 150mM NaCl, 15mM sodium citrate). A piece of Hybond-N⁺ (*Amersham International plc*) nylon membrane pre-soaked in neutralisation solution was then carefully placed on top of the gel to avoid air bubbles, and the position of the

wells on the gel marked with a pencil. 10 more pieces of 3MM Whatman paper pre-soaked in 20 x SSC were placed on top of the nylon membrane and some dry paper towels were placed over them with a weight on top. DNA was allowed to transfer on the membrane overnight and the next day the membrane was carefully removed, washed in 6 x SSC and dried for 30 minutes at room temperature before crosslinking the DNA to the membrane using a UV Stratalinker 2400.

2.2.15.2 Radiolabelling of DNA probe

The fragment of DNA (~300bp) to be used to identify the recombinant protein in the newly made viruses, was purified from the plasmid (§2.2.6). Labelling of the fragment was then carried out based on the random labelling reaction described by (Feinberg & Vogelstein, 1983) using the Ready-to-Go™ DNA labelling Beads (-dCTP) (*Amersham Biosciences*) as specified by the manufacturer. DNA was initially denatured (3 minutes 100°C) and added to the reaction mix bead (containing dATP, dGTP and dTTP as well as DNA polymerase) together with 5µCi of $\alpha^{32}\text{P}$ -dCTP to reach a final volume of 50µl. The labelling reaction was then carried out at 37°C for at least 30 minutes before purifying the mix of unincorporated radioactivity by passing through a G50 Sephadex column (*Amersham Bioscience*). The purified probe was then made single stranded by heating at 100°C for 5 minutes and then immediately cooled on ice for 2 minutes. This probe was then added to the membrane.

2.2.15.3 Hybridisation of the southern blot membrane

The membrane obtained from transfer of DNA (§ 2.2.15.1) were pre-hybridised for 1 hour at 65°C in 30ml of pre-hybridisation solution (5 x SSC, 5 x Denhardt's reagent (*Sigma*), 0.5% (v/v) SDS in ddH₂O) to which 100µg/ml of denatured (95°C 5minutes, ice 2 minutes) herring sperm DNA (*Sigma*) were added. After at least 1hour of pre-hybridisation, 20ml of solution were removed and the radiolabelled probe (§ 2.2.15.2) was added and left to hybridise to the membrane overnight at 65°C.

Following hybridisation, the membrane was washed three times with solutions of decreasing stringency. An initial 5minutes wash with 2 x SSC, 0.5% SDS was

followed by a 15minutes wash with 2 x SSC, 0.1% SDS and a final 30minutes wash with 0.1 x SSC, 0.1% SDS. The membrane was then wrapped in cling film, exposed to Kodak X-Omat AR x-ray film (*Sigma*) and developed after varying lengths of time.

2.2.16 Protein isolation and analysis

Resolving gel Buffer

1.5M (18.15g) Tris
0.013M (0.4g) SDS
in H₂O (100ml)
pH to 8.9 with HCl

Stacking gel Buffer

0.425M (5.1g) Tris
0.013M (0.4g) SDS
in H₂O (100ml)
pH to 6.7 with HCl

10 X Running Buffer

0.247M (30g) Tris
1.918M (144g) Glycine
0.0658M (19g) SDS
Dissolved in H₂O (1L)

Boiling mix

1ml stacking buffer
0.8ml 25% SDS
0.5ml 2-mercaptoethanol
bromophenol blue

2.2.16.1 Protein extraction

Monolayers of 10⁶ BHK or Mam49 cells were washed twice with 1ml of HBSS and lysed adding 1ml of boiling mix and swirling using a bent pipette plastic tip. The samples were then immediately placed at -20°C until required. For extraction from dendritic cells, 10⁶ cells were spun down (3 minutes 238g -1500rpm) and after removal of supernatant the pellet of cells was washed twice with 1ml of HBSS before being lysed by addition of 1ml of boiling mix. These were stored at -20°C until used.

Extraction of protein for samples to be tested for expression of influenza NP were carried out using the urea method that is usually used for transmembrane proteins that does not require boiling of the samples. The medium was removed from 10⁶ cells either monolayer or DCs in suspension (in this case by centrifuging the cells 3minutes 238g and removing the supernatant) and cells washed in 1ml of HBSS by centrifuging for 3minutes at 425g (2000rpm). The pellet was then resuspended in 100µl of urea extraction buffer (48% (v/v) SDS, 2M Urea, 8% sucrose to which

protease inhibitors (complete Mini-Roche) were added fresh immediately before use). The sample was passed 10 times through a 21 gauge needle (*BD Microlance*) and syringe to disrupt cellular membranes, and immediately frozen at -20°C until required.

2.2.16.2 SDS Polyacrylamide gel electrophoresis (SDS-PAGE)

Protein MW Range (kDa)	12-45	14-60	16-70	60-200
Acrylamide concentration	15%	12%	10%	5%

Table 2.3: Required acrylamide concentration of resolving gel according to the molecular weight of the protein.

Resolving gel (10%)	Bis-Acrylamide (30:1.5)* Resolving gel buffer 10% (w/v) APS TEMED** ddH ₂ O 10M Urea***	8ml 6ml 150μl (300μl in urea gels) 15μl (30μl in urea gels) 11ml (6ml in urea gels) 5ml
Stacking gel (5%)	Bis-Acrylamide (30:1.5) Stacking gel buffer 10% (w/v) APS TEMED** ddH ₂ O 10M Urea***	2ml 3ml 150μl 15μl 7ml (4.5ml in urea gels) 2.5ml

Table 2.4: Composition of resolving and stacking gel of SDS-PAGE gels

*Bis:N,N'-Methylene-bis-acrylamide

**NNNN-Tetraethylethanediamine (*Bio-Rad*, Hemel Hempstead, Herts). This is added last as is responsible for polymerisation

*** Only added in urea gels

22ml of resolving gel were poured between two glass plates separated by spacers and isopropanol was overlaid to ensure it is straight and to remove air bubbles. The density of the resolving gel (varying amount of acrylamide) was decided

based on the size of the protein to be identified (table 2.3). For detection of NP (~60KDa) and PyCS (~60KDa) gels were made at 10%, whereas gels to detect PySSP2 (~140KDa) gels were 5%. This was left to set before removing the isopropanol, washing twice with dH₂O and drying the excess with 3MM Whatman paper. The 5% stacking gel was then poured on top of the set resolving gel, an appropriate comb was inserted into it to allow formation of wells and was left to polymerise. Urea based gels were assembled in the same way but differing slightly in the composition of the buffers (table 2.4).

Upon complete polymerisation, the comb was carefully removed from the stacking gel and the total protein samples were separated into polypeptide units by SDS-polyacrylamide gel electrophoresis according to the method of (Laemmli, 1970). Samples were prepared immediately before loading on the gel. Samples frozen in boiling mix were thawed and heated at 95°C for 5 minutes and then placed on ice for 10 minutes before being loaded onto the gel (whole sample or 50µl were loaded on the gel).

Samples extracted in urea extraction buffer were thawed and mixed with an equal amount of sample buffer (urea extraction buffer with 350mM 2-mercaptoethanol added and bromophenol-blue). This mixture was heated to 37°C for 10 minutes and the whole sample was loaded on a urea gel. A coloured molecular weight protein marker (Rainbow marker) (*Amersham Biosciences*) was also loaded on the gels (10µl). SDS-polyacrylamide gels were prepared in a vertical gel electrophoresis separation system (*Owl*) and run in 1x running buffer at 40mA/gel until good separation was obtained between the coloured molecular weight markers comprising the expected band.

2.2.16.3 Transfer of protein to nitrocellulose by western blotting

Proteins separated on SDS-PAGE gels were transferred onto nitrocellulose membranes (Hybond-C) (*Amersham International Plc*) using a wet-transfer method described by (Towbin et al., 1979). The gel, nitrocellulose membrane and six sheets of 3MM Whatman paper were soaked in transfer buffer (50mM Tris, 380mM glycine, 0.1% (w/v) SDS and 20% (v/v) methanol). The gel and nitrocellulose membrane were then placed in between the filter paper in a Trans-

Blot™Cell (*BioRad*) according to manufacturer's instructions. Protein band transfer onto the membrane was carried out overnight at 4°C at 5V. Upon completion of transfer onto the nitrocellulose membrane this was stained for immunodetection of the protein.

2.2.16.4 Immunodetection of proteins on nitrocellulose membrane

The nitrocellulose membrane with the transferred protein bands were blocked by incubating in blocking solution (5% (w/v) skimmed milk, 0.1% (v/v) polyoxyethylene sorbitan monolaurate (Tween-20) made up in 1 x PBS) for at least 1 hour shaking at room temperature. The membrane was then incubated with primary antibody diluted in 2% (w/v) skimmed milk, 0.1% (v/v) Tween-20 in 1 x PBS for 2 hours at room temperature with vigorous shaking. The antibody dilutions used are shown in table 2.1. Unbound antibody was then removed and the membrane rinsed with three 10 minutes washes with 1 x PBS 0.1%(v/v) Tween-20 at room temperature with constant shaking. The washed membrane was then incubated in the dark with the appropriate secondary anti-IgG horseradish peroxidase (HRP) conjugated secondary antibody diluted in 2% (w/v) skimmed milk made up in 1 x PBS 0.1% (v/v) Tween-20 shaking for 1 hour at room temperature. The unbound HRP antibody was then removed from the membrane by three washed for 10 minutes each with 1 x PBS 0.1% (v/v) Tween-20 at room temperature shaking. The bound HRP was visualised using the Enhanced Chemilluminescence system (ECL™) (*Amersham International*) according to the manufacturer's instructions. Exposure times varied between 2 seconds and 20 minutes, according to the strength of the signal.

2.3 Tissue culture

Tissue culture manipulations were carried out under sterile conditions in a laminar flow safety cabinet class II (*Heraeus*) using standard aseptic techniques. Cells were maintained in incubators (*Heraeus*) at 37°C with 5%CO₂ in a humidified atmosphere. Primary cell preparations were obtained using separate cabinets and incubators from cell lines.

2.3.1 Heat inactivation of foetal calf serum (FCS)

FCS was heat inactivated by placing at 65°C for 45 minutes

2.3.2 Propagation of mammalian cell lines

BHK-21: Baby Syrian Hamster Kidney 21 clone 13 cells (ECACC number: 85011433).

These cells were grown in full growth medium (FGM): Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum (FCS) and 100U/ml penicillin and streptomycin. The cells were passaged when reaching a 80% confluency by washing with Hanks Balanced Salt Solution (HBSS) at room temperature and then incubated with thin layer of 10% (v/v) trypsin in versene until detachment of cells. This was stopped by addition of FGM and the cells split at a ratio of 1:20 with FGM into fresh flasks.

27/12/M:4 (Mam49) (Thomas et al., 1999): These are a BHK based cell line transfected with plasmids encoding ICP27, ICP4, and equine VP16. These cells are grown in same medium as BHK cells but supplemented with 800µg/ml of Geneticin-G418 sulphate (*Gibco*) and 750µg/ml of Zeocin (*CAYLA*).

A20 cells: Balb/c cell lymphoma line (ATCC # TIB-208). These are B lymphocyte cells from reticulum cell sarcoma A20 cells were maintained in RPMI 1640 supplemented with 2mM L-Glutamine, 10% heat inactivated FCS, 100U/ml penicillin and streptomycin

P815-1-1 cells: Mouse DBA/2 mastocytoma cell line (ECACC number: 85011406). These cells were propagated in RPMI 1640 supplemented with 2mM Glutamine, 100U/ml penicillin and streptomycin and 10% heat inactivated FCS. The cells were split when reaching ~80% confluency by washing the cells with HBSS and pelleting by centrifugation followed by 1:10 replating.

2.3.3 Cell line storage

For long term storage, cells were taken in log phase. 0.5ml of cell suspension was mixed with 0.5ml of ice-cold freezing mixture (85%FCS, 20% dimethylsulphoxide (DMSO)) and placed in 1.5ml cryovials. These were slowly cooled to -70°C for 24hr before being stored in liquid nitrogen. For recovery, cells from one vial were rapidly thawed, and transferred immediately to 10ml of fresh FGM specific for that cell type and incubated overnight. The FGM was replaced the next day with 10ml of fresh appropriate FGM.

2.3.4 Preparation of primary human dendritic cells from blood or buffy coat

This protocol follows the one described by Romani et al 1994 and Morse et al 1997

100ml of peripheral blood mononuclear cells (PBMCs) were collected from healthy donors using syringes coated with heparin anticoagulant. The blood was added to 80ml of HBSS and this carefully layered onto 17ml of lymphosep (*Fisher scientific*) previously placed in 50ml tubes (*Falcon*). After centrifugation for 30minutes at room temperature 486g (1600 rpm) without break, the mononuclear cells in the interface was carefully removed using a Pasteur pipette and washed with HBSS (centrifuge 486g for 10minutes at room temperature). The cells were pulled together and washed twice with HBSS centrifuging for 5minutes at 372g (1400 rpm) at room temperature. The pellet was then lysed of red blood cells by addition of 10ml of red blood cell lysis buffer (*Sigma*) for 5 minutes. Cells were then washed twice more with HBSS and then resuspended in 18ml of complete medium (RPMI 1614 with 10% heat inactivated FCS, 100IU/ml of penicillin/streptomycin). Cells were plated in six-well plates at 3ml/well and incubated 1.5 hours at 37°C , 5%CO₂. Non adherent cells were placed into a fresh tube and washed in HBSS centrifugating at 372g (1400rpm) for 5minutes and plated again. The adherent cells after both steps were replenished with fresh FGM

supplemented with hGMCSF (*Insight Biotechnology*) at 0.1µg/ml and IL4 (*Insight Biotechnology*) at a concentration of 0.05µg/ml.

On day 8 the cells were depleted of other cell types. Non adherent cells were harvested with gentle washing, and then pelleted at 4°C for 3mins at 321g (1300 rpm). The pellet was washed with ice cold HBSS and the supernatant discarded. The DC pellet was resuspended in 2ml of pre-chilled complete RPMI medium. The cells were then placed on ice. For each million cells these were incubated with 1µl of anti-CD19 (TCSN); 2µl anti-CD3(TCSN), and 1µg/ml anti-CD2 (*Insight Biotech*). The mixture was placed on a rotating mixer in cold environment and incubated for 30min. Cells were then washed with ice cold HBSS and resuspended in 2ml of complete medium and then added to the dynabeads (dynabeads M450, *Dynal*) (10µl of beads/million cells) previously prepared. Beads and cells were then incubated on a rotating mixer at 4°C for 30mins. DCs were then depleted by removing the supernatant after placing the suspension mix in contact with a magnet. The purified DC were then counted, resuspended at the required concentration with FGM and placed on ice for immediate use.

2.3.5 Preparation of mouse bone marrow dendritic cells

Bone marrow dendritic cells were derived from 4-6 weeks old female BALB/c mice and cultured following slightly modified protocol by Lutz et al (Lutz et al., 1999). The cells from the mice tibiae and femur were flushed and washed twice with wash buffer (HBSS 2%FCS) by centrifuging 5minutes at 372g (1400 rpm) at room temperature. The red blood cells present in the pellet were then lysed by resuspending in 3ml red blood cell lysis buffer (*Sigma*) and incubating for no longer than 5 minutes at room temperature. The cells were then washed twice with wash buffer before being resuspended into 1ml of complete medium (Iscove's modified Dulbecco Medium, 10% heat inactivated FCS, 100U/ml penicillin and streptomycin, 50µM 2-mercaptoethanol [*Gibco*]). Cells were counted and resuspended at a concentration of 6×10^5 cells/ml in complete medium supplemented with 10ng/ml mouse recombinant GMCSF (mGMCSF) (*AutogenBioclear*) to allow DC differentiation. The cells were plated on six-well plates and kept in humidified chamber 37°C 5%CO₂.

On day 3 half the medium was replaced with fresh complete medium supplemented with mGMCSF taking care not to disrupt clumps of semi-adherent cells. The cells were then cultured for a further 4 days. On day 7 non adherent and slightly adherent cells were harvested and concentrated using lymphosep (*Harlan SeraLab*). In 15ml polystyrene screw cap tubes (*Nunc*) cells were overlayed onto 3ml of lymphosep and spun at 486g (1600rpm) for 20min without break. The interfase containing the dendritic cells (DC) was then removed and washed twice with wash buffer. This step helps to obtain a cleaner population of cells (Lappin et al., 1999). Cells were resuspended in 1ml complete medium, counted and used immediately.

2.3.6 Preparation of splenocytes from mouse spleen

Cultures of fresh splenocytes from immunised mice were set up to be tested in an ELISPOT assay for presence of an antigen specific T cell response by the mice. Because the number of reactive cells may be relatively low and therefore causing detection problems, these cells can be amplified over non antigen targeted cells, by culturing them in presence of the specific peptide.

Mouse spleen were removed, disaggregated by using a syringe stuffer and passed through a 70µm nylon cell strainer (*Falcon, BD biosciences*) by flushing with 10ml wash buffer (HBSS 10% heat inactivated FCS). A different strainer and syringe stuffer was used for each spleen. Cells were placed into a 50ml tube and spun at 321g (1300 rpm) 4°C 5 minutes. The pellet was then washed once with wash buffer and treated with 5ml of red blood cell lysis buffer (*Sigma*) for 5 minutes. The reaction was stopped by addition of 10ml wash buffer and washing cells twice. The final pellet was resuspended in 2ml of complete medium (RPMI 1614, 10% heat inactivated FCS, 50µM 2-mercaptoethanol, 100U/ml penicillin and streptomycin) containing 10µM of the appropriate peptide for restimulation (§ 2.3.13). Cells were counted, plated at a concentration of 2×10^6 cells/ml in six-well plates and placed in a humidified chamber at 37°C 5%CO₂ for 7 days. On day 7 splenocytes were harvested and washed twice with HBSS by centrifuging 5minutes at 321g (1300rpm) 4°C. The pellet was resuspended in 2ml of complete medium and cells were counted and used immediately.

2.3.7 DNA transfection for transient expression

DNA was transfected into mammalian cell lines either to check a plasmid for gene expression or to obtain recombination for the construction of new viruses (§2.3.9)

DNA transfections were performed based on the calcium phosphate mediated method as previously described (Stow & Wilkie, 1976) and used supercoiled plasmid DNA.

Hebes trasfection buffer: 140mMNaCl, 5mMKCl, 0.7mMNa₂HPO₄, 5.5mMD-glucose, 20mMHepes, pH7.05 with NaOH. This was filter sterilised through a 0.2µm filter and stored at 4°C.

Cells were grown to 80% confluency in six-well plates. Two tubes were set up:

Tube1: 31µl 2McaCl₂, 10µg plasmid DNA, 20µg herring sperm DNA (phenol/chloroform extracted).

Tube 2: 400µl Hebes transfection buffer

The contents of tube 1 were carefully mixed by gentle pipetting and then added to tube 2 drop-wise with constant mixing. The mixture was left at room temperature for 40minutes to allow DNA precipitation. The media was then removed from the monolayer of cells and the mixture of precipitated DNA was carefully added to the cells. These were incubated at 37°C 5%CO₂ for 30min gently shaking every 10min to avoid drying out of the cells. Following this incubation 1ml of FGM was added to the cells and these placed back in the incubator 37°C 5%CO₂ for 4-6hours prior to DMSO shock. A 20% DMSO sterile solution was prepared in Hebes transfection buffer. After removal of the media from the cells, these were gently washed twice with 1ml of FGM and 1ml of 20% DMSO was added onto the cells for no longer than 90seconds. The DMSO solution was then removed and the cells washed twice with 2ml of FGM. A final 2ml of FGM was added to the cells and then incubated at 37°C 5%CO₂ for 48hr.

2.3.8 Virus infection of monolayer cells

The virus was diluted in a minimal final volume of SFM that would allow for overlay of the whole cell surface (eg. 500µl for a six-well). This was overlayed onto the monolayer from which the medium had previously been removed, and

the cells incubated at 37°C, 5%CO₂ for 1hour before overlaying with FGM supplemented with HMBS when required.

2.3.9 Construction of recombinant HSV-1 virus

2.3.9.1 Virus transfection

The principle by which construction of recombinant viruses are formed is illustrated in figure 2.1. To ensure maximal efficiency of viral transfection and infection, wells, flasks and plates of appropriate cells were prepared without selection present in the media.

Transfections to obtain a recombinant viruses were carried out similarly to plasmid transient transfections as described in section 2.3.7 except 10-30µg of purified viral DNA (§2.3.9.6) was added to tube 1. For construction of viruses with mutated VP16 the media was supplemented with 3mM hexamethylene bisacetamide (HMBA) in order to induce immediate early gene transcription (McFarlane et al., 1992). Homologous recombination transfections were left for 3-5 days until complete cytopathic effect (CPE) was observed. The well was then harvested and freeze-thawed. The harvested cells were then titred out in a plaque assay (§2.3.9.2) and the efficiency of recombination determined by assaying for recombinants containing the required reporter gene.

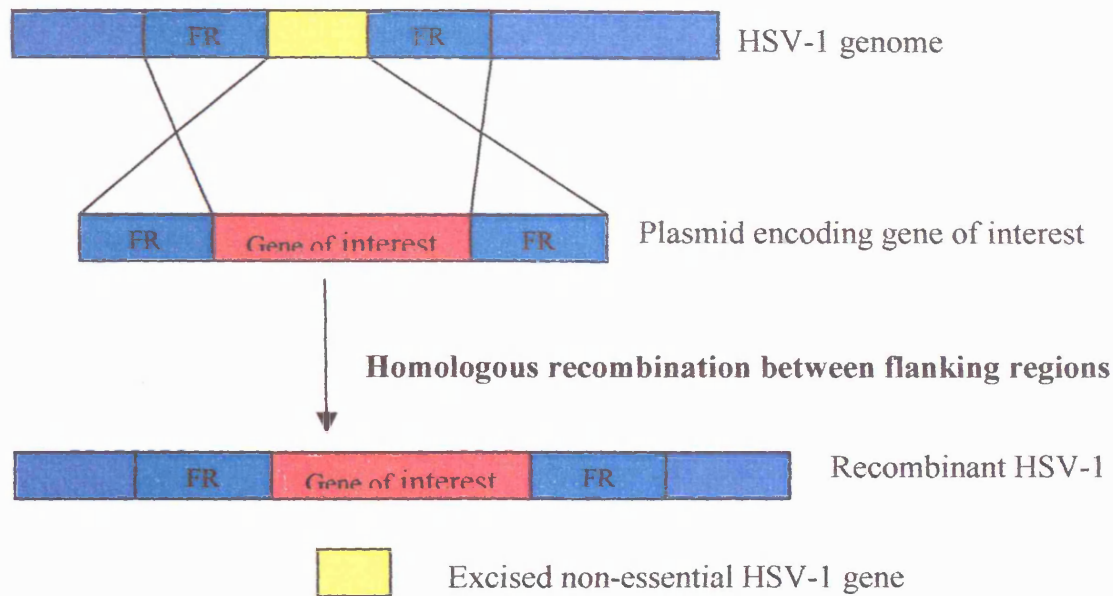


Figure 2.1: Homologous recombination

Schematic representation of the homologous recombination that needs to take place for the construction of recombinant HSV viruses. A plasmid containing the gene of interest (either a therapeutic gene or GFP) between the two viral flanking regions (FR) of the gene to be replaced in the HSV genome is transfected together with the viral DNA. Homologous recombination takes place between the flanking regions excising the viral gene and replacing it with the one from the plasmid. This forms the recombinant HSV virus that is then purified.

2.3.9.2 Plaque assay

BHK or Mam49 cells were grown to 80% confluency in six-well plates. A 1:10 serial dilution of the virus preparation was prepared in DMEM without FCS (serum free media, SFM) to a volume of 100 μ l. The 100 μ l were added to 400 μ l of SFM placed on the cells, and these were incubated for 1hr at 37°C, 5%CO₂. After this incubation 2ml of 1:2 (v/v) FGM: 1.6% (v/v) carboxymethyl cellulose (CMC) were added to each well. When using viruses with mutated VP16, 3mM HMBA was also added as well. Cells were then incubated for 48hours at 37°C, 5%CO₂ and the number of plaques in each well were counted in order to determine the virus titre in plaque forming units/ml.

2.3.9.3 Detection of GFP expression

Cells expressing GFP could be directly visualised using an inverted fluorescent microscope at a wavelength of 520nm.

2.3.9.4 Purification of recombinant virus by plaque assay

The harvested recombinant viral transfections were freeze-thawed to disrupt the cells allowing release of virus. The transfection was then plated in a plaque assay serial dilution. When using the plaque assay to purify a newly made recombinant virus, this also determined efficiency of infection by looking at the number of plaques expressing the recombinant protein under normal and UV light with the appropriate filter to visualise green, red, or white (without insert) plaques using an inverted fluorescent microscope (*Nikon Eclipse TE200*). Plaques expressing the reporter genes of interest were picked from the monolayer using a P20 *Gilson* micropipette (set at 18 μ l) and transferred to an eppendorf tube containing 100 μ l of SFM. The plaque suspension was then freeze-thawed and plated out for further purification. The plaque purification process was repeated until the plaques formed were only those containing the recombinant gene and no parent virus. Each well was then harvested and used as stock to propagate the virus at a larger scale.

2.3.9.5 Production of high titre stock of recombinant virus

100 μ l of the harvested purified plaques stock was used to infect an 80% confluent 175cm² flask of the appropriate cell line and medium without selection. Infection

of flasks was carried out initially incubating the infecting virus in 5ml of SFM on the cells for 1 hr, after which 20ml of FGM with HMBA added when needed. This was incubated at 37°C, 5%CO₂ until achievement of complete CPE when the flask was harvested and frozen. This sub-master stock was titred and used to further propagate the virus into high titre stocks.

Ten 500cm² tissue culture plates were seeded with the appropriate cell line in 50ml of FGM not containing selection. At 90% confluency, cells were infected at an MOI of 0.05 (3x10⁶ pfu/ml) with the sub-master stock, in a total volume of 50ml of fresh FGM without selection but supplemented with HMBA when necessary. The plates were then incubated at 37°C, 5%CO₂ for 2-4 days until complete CPE was observed. The whole plate was then frozen at -80°C and then thawed to release the virus from the cells. After thawing at room temperature, cellular debris was removed by centrifugation at 2330g (3500rpm) for 45 minutes at 4°C. The supernatant was then passed through a 5µm filter, followed by a 0.45µm filter and the viral particles were then pelleted by centrifugation at 21,859.2g (12,000rpm) for 2 hours at 4°C. The supernatant was discarded and the pellet resuspended in 200µl of HBSS. The resuspended pellet was then sonicated for 5 times 10 seconds in a water bath sonicator and chilled on ice between sonications. The virus was then aliquoted and stored in liquid nitrogen. Virus titre was tested by plaque assay (§2.3.9.2).

2.3.9.6 Viral DNA extraction

In order to obtain viral DNA for Southern blot analysis, viral extractions were carried out from one well of a six-well plate infected, and at full CPE. Alternatively, when isolating DNA to be used in the construction of recombinant viruses, this was done from a 175cm² flask of infected cells at complete CPE.

The medium was removed from the monolayer, the cells were washed gently with 1ml of HBSS (5ml in the case of 175cm² flask) before adding 1ml (or 6ml for the 175cm² flask) of DNAzol (*Helena Biosciences*). This was swirled until the entire surface area was covered with solution. The lysate was added to a 15ml Falcon tube and overlaid with 3ml of absolute ethanol. This mixture was gently swirled to allow DNA precipitation initially as strings at the interface and subsequently as a

lump. The DNA was removed with a pipette tip and washed in 3ml of 75% ethanol twice. DNA was finally placed into a 1.5ml tube and centrifuged 1 minute at 2653g (5,000 rpm) to remove the excess ethanol. The pellet was air dried at room temperature for 5 minutes and resuspended in 200 μ l (or 1 ml for the 175cm² flask) of 8mMNaOH freshly made. This DNA was then incubated at 4°C on a rotating platform overnight to allow good homogenisation, and then neutralised with 23 μ l (or 115 μ l of the 175cm² flask extraction) of 0.1M HEPES. Viral DNA was then stored at -20°C.

2.3.10 Transduction of dendritic cells with HSV-1

The required number of cells (5×10^6 - 10^6) was placed in a 15ml tube and centrifuged 372g (1400rpm) for 5 minutes at room temperature. The supernatant was decanted and the cells resuspended in 100 μ l of serum free medium and high titre purified virus were added at MOI of 1 unless otherwise specified in the text. The cells were incubated for 1hour at 37°C, 5%CO₂ after which complete medium was added supplemented with mGMCSF for mouse cells or hGMCSF and IL4 for human DCs to achieve concentration of 5×10^5 cells/ml and were plated in 24 well plates overnight at 37°C, 5%CO₂. The next day cells were harvested and used for FACS analysis.

When transducing mouse bone marrow dendritic cells for in-vivo injection, these were not incubated with medium containing cytokines because they were harvested 4 hours after infection. Cells were pelleted (5 minutes 427g -1500 rpm) and then washed twice with HBSS and counted before being resuspended in HBSS in an appropriate volume for injection.

For stimulation of maturation DCs were treated with lipopolysaccharide (LPS) (*Sigma*). LPS was added with the complete medium at a concentration of 100ng/ml for human cells and 5 μ g/ml for mouse bone marrow derived DC.

2.3.11 Trypan blue exclusion assay

Cell survival and most cell counting were made by trypan blue exclusion assay. With this test dead cells are identified because of they take up the blue dye. 10 μ l of cell preparation were mixed to an equal volume of 0.04% trypan blue solution

and then loaded on a haemocytometer counting chamber. The live and dead cells were counted on a 4 x 4 square grid, this count was multiplied by a factor of 2×10^4 to obtain counts of cells/ml.

2.3.12 HSV-1 Growth curves

24-well plates with 80% confluent BHK or Mam49 cells (as specified by the text) were infected at MOI 0.01 and 0.001 with the virus (§ 2.3.8) in duplicate for each time point. These were harvested at 0, 2, 4, 8, 20, 24, 48 hours post infection. Cells were disrupted by freeze-thawing and virus yield was assessed by plaque assay (§ 2.3.9.2). All titrations and infections were carried out in duplicate.

10^5 human DCs were transfected at MOI of 1 (§ 2.3.10) and frozen at 0, 10, 20, 24, 36, 48, 72 and 96 hours post-infection. After thawing of the samples to release the virus the yield was determined by titration of BHK cells. Similarly, 2×10^5 or 2×10^6 mouse bone marrow dendritic cells were transfected at MOI of 1 with the appropriate virus and samples were frozen at 0, 4, 8, 20, 24, and 48 hr post infection. After thawing all samples were titrated in duplicate to determine the yield of the virus by plaque assay.

2.3.13 Peptide pulsing of cells

Bone marrow derived dendritic cells

Cultured DCs were stimulated to mature overnight with LPS (§ 2.3.5). The mature DC were harvested and placed in a screw cap tube and after washing twice with serum free medium (Isocove's Modified Dulbecco medium) by centrifugation at 427g (1500rpm) 5 minutes the cell pellet was resuspended in 100µl of serum free medium. The antigen diluted in the appropriate solvent (table 2.5) was added to the cells in the tube at a concentration 10µM considering as volume that in which the cells would have been plated (6×10^5 cells/ml). All peptides are nine-mer since this is the size of peptides that fit the MHCI binding cleft. DCs were pulsed for 45 minutes in a humidified chamber at 37°C, 5%CO₂ after which they were washed twice with HBSS before being resuspended in appropriate volume for in-vivo injection.

Purified peptides were obtained from *Invitrogen*. When their polarity required the peptides to be dissolved in DMSO this was added in minimal amount to ensure minimal disruption of cells and the volume was made up with HBSS.

Protein	Peptide sequence	Amino acid position	Solvent
NP	TYQRTRALV	147-155	HBSS
CS	SYVPSAEQI	280-288	DMSO
SSP2	KYIFVVLLL	8-16	DMSO

Table 2.5: Table indicating the sequence, position within the protein sequence and the appropriate solvent for the H2K^d CD8⁺ peptides used in this project.

A20 cells

A20 cells were pulsed with antigen peptides to act as antigen presenting cells in the set up of ELISPOT assays (§ 2.4.4). ELISPOTS were set up using 100µl of A20 10⁶/ml per well. Cells were pulsed at 20µM to account for the addition of one volume of responder cells therefore resulting in final pulsing at 10µM.

A20 cells from an 80cm² flask in culture were harvested and spun down by centrifugation 5 minutes at 427g (1500 rpm). The cells were then washed twice with HBSS and the pelleted cells resuspended in 5ml of RPMI 1614 (serum free). These were counted and the number of required cells were spun down and resuspended in 200µl of RPMI 1614 to which enough peptide to achieve a concentration of 20µM in final volume was added. Cells were mixed well and the tube placed at 37°C, 5%CO₂ for 30minutes before adding the amount of serum free medium required to achieve a cell concentration of 10⁶cells/ml.

2.4 Immunological assays

2.4.1 Flow cytometry

Dendritic cells were analysed for expression of surface molecules by staining with fluorescent antibodies for flow cytometric quantitation.

2×10^5 previously counted DCs were placed in 96well round bottom plates and washed twice in 300 μ l FACS buffer (15mM EDTA, 30mM NaN₃, 1% (w/v) bovine serum albumin (BSA), in 0.1M phosphate buffer PB), by centrifuging at 427g (1500rpm) 3minutes at 4°C. The cells were initially fixed by incubation in 100 μ l of 4% (w/v) paraformaldehyde for 20minutes on ice. For intracellular staining, after three washes with 300 μ l of FACS buffer at 427g (1500rpm) 3minutes at 4°C, the cells were permeabilised with 100 μ l of freshly prepared 0.3% (w/v) saponin in 0.1MPBS for a further 45minutes on ice and then washed two times. The pelleted cells were resuspended in 100 μ l of FACS block solution (0.1M PBS, 10%FCS) left on ice for 10minutes. Cells were then spun down 3 minutes at 427g (1500rpm) 4°C to remove the blocking solution, and the pellet resuspended in 100 μ l of antibody dilution (table 2.1) in block solution, this was left on ice for 1hour. The cells were then pelleted by centrifugation 427g (1500rpm) for 3minutes at 4°C and the supernatant carefully removed. The pellet was then washed three more times with 300 μ l of FACS buffer spinning at 427g (1500rpm) for 3minutes at 4°C.

100 μ l of the secondary antibody, conjugated to a fluorochrome (or biotin conjugated for anti-CD11c) was then added at the appropriate dilution (table 2.1) in blocking solution and incubated on ice in the dark for 30minutes then followed by three washes with FACS buffer. For mouse bone marrow dendritic cells anti-CD11c this was then followed by a staining with a third antibody conjugated to streptavidin and to a fluorochrome. Cells were washed three more times with 300 μ l of FACS buffer by centrifugation at 427g (1500rpm) 4°C for 3 minutes and the pellet resuspended in 300 μ l of FACS buffer for analysis. The cells were transferred into Falcon FACS tubes (Becton Dickinson) and run on a FACScalibur (*Beckton Dickinson*). The right population was identified based on size and granularity and analysed for detection of specific fluorochromes.

2.4.2 Mouse GMCSF Enzyme Linked Immunosorbent Assay (ELISA)

The supernatant from BHK, Mam49, or 10^6 DCs cells infected with viruses expressing mGMCSF or, as negative control, viruses not expressing mGMCSF, were collected and stored at -20°C .

50 μl of sample or of standard were then tested for presence of mGMCSF using the Granulocyte-Macrophage Colony Stimulating Factor [(m)GM-CSF] mouse, Biotrak ELISA System (*Amersham Biosciences*) following the procedure suggested by the manufacturer. Briefly, samples were incubated at 37°C , 5%CO₂ in plates pre-coated with anti-GMCSF antibody, the wells were then washed and incubated with anti-GMCSF antibody conjugated to HRP before washing again and developing the reaction with TMB substrate solution. The reaction was stopped using 0.18M sulphuric acid, and the plate read for chemiluniscence at 450nm using a plate reader (*OpsysMR-Dynex*). Samples were always run in duplicates

Standard curves were then obtained using the values from the standard samples, and values extrapolated from this one.

2.4.3 Serum antibody Enzyme Linked Immunosorbent Assay (ELISA)

Coating agent	Specification
Purified NP protein	Influenza virus, type A (H3N2-Shangdon) <i>Advanced ImmunoChemical</i>
H2K ^d B epitope PyCS	(QGPGAP) ₃
H2K ^d B epitope PySSP2	(NPNEPS) ₃

Table 2.6: Coating agent used for set up of ELISA for detection of antibodies in mouse serum

These ELISA assays were set up in 96-well Nunc-MaxiSorp plates. Each well was incubated overnight at 4°C with 50 μl of the appropriate dilution (§ chapter 7) of

peptide or protein in 1x PBS (table 2.6). Excess coating reagent was removed by washing the wells three times with 200µl of ELISA wash (0.05%(v/v) Tween-20 in 1 x PBS) and these were then blocked with 100µl of ELISA block (3% (w/v) Milk, 0.05%(v/v) Tween-20 in 1 x PBS) for 1hour at room temperature shaking. The block was removed and the wells washed three times with 200µl of ELISA wash before adding 50µl of serial dilution of mouse serum (§ 2.5.6) in ELISA block, or anti-protein antibody at the required concentration (Table 2.1) as positive control. These were left shaking at room temperature for 2hours and then removed and the wells washed three times with 200µl of ELISA wash. The secondary antibody anti-mouse HRP conjugated (*DAKO*) diluted 1:1000 in ELISA wash was then added at 100µl per well and incubated shaking in the dark at room temperature for 1hour. Wells were again washed three times with 200µl of ELISA wash and then developed in the dark with 100µl of TBS substrate/well at room temperature for 30minutes and the reaction stopped with 100µl of 0.18M sulphuric acid. Plates were read at 450nm in a plate reader (*OpsysMR-Dynex*).

2.4.4 Mouse IFN γ Enzyme-linked immunospot (ELISPOT) assay

2.4.4.1 ELISPOT Assay

IFN γ ELISPOTS were set up to determine the presence of specifically activated primary/memory T cells in immunised mice. The Mouse IFN- γ ELISPOT set (*BD Biosciences*) was used according to manufacturer instructions.

Splenocytes that had been restimulated in vitro with the appropriate peptide (§ 2.3.6) were harvested and placed in 50ml Falcon tubes. The cells were washed twice with HBSS by centrifuging at 321g 5minutes at 4°C, resuspended in full growth medium and counted. Splenocytes were concentrated at 10^6 cells/ml in FGM. Stimulator A20 cells were pulsed with the appropriate peptide (§ 2.3.13) and resuspended at a final concentration of 10^6 cells/ml. The ELISPOT assay was then assembled in plates (either from the kit, or Millipore MultiScreen Assay System 96 well plates 45µm- *Millipore*) previously blocked for 1hour with 200µl of FGM. Three wells per sample were used. One well contained 100µl corresponding to 10^5 splenocytes/well and two wells contained 100µl of 5×10^4 splenocytes/ well. Splenocytes were incubated with either 100µl of FGM, 100µl

of 10^6 cells/ml non pulsed A20 cells, or 100 μ l of 10^6 cells/ml peptide pulsed A20 cells. As positive control, splenocytes were also incubated 100 μ l of FGM containing 200ng/ml Phorbol 12-myristate 13-acetate (PMA), 20 μ M Ionomycin. Plates were left at 37°C, 5%CO₂ 24hr and then developed according to manufacturers instructions. Briefly, wells were washed three times with 350 μ l/well of ddH₂O followed by two washes with 350 μ l/well of 0.05% (v/v) Tween-20 in PBS. 60 μ l/well of detection anti-IFN γ antibody were added diluted appropriately in dilution buffer (10% (v/v) FCS in PBS) and incubated at room temperature for 2 hours or longer at 4°C. The detection antibody was then discarded and the wells washed three times with 350 μ l/well of 0.05% (v/v) Tween-20 in PBS before adding 60 μ l/well of avidin-HRP antibody appropriately diluted in dilution buffer. This was incubated for 1 hour at room temperature. After four washes with 200 μ l of 0.05% (v/v) Tween-20 in PBS, and two washes with 200 μ l/well of PBS, 100 μ l of substrate were added to each well. The substrate was prepared as 333 μ l of 10mg/ml AEC (3-amino-9-ethyl-carbazole) in DMF (N,N-Dimethylformamide) to 10ml 0.1M Acetate filtered through 0.45 μ m filter 5 μ l of H₂O₂ were added per 10ml of substrate. The substrate was left for a maximum of 1 hour depending on the speed of spots development and the reaction was stopped by washing extensively with water. The plate was then air dried and spots counted on an ELISPOT reader (AID) using AID3.2 programme.

2.4.4.2 Analysis of ELISPOT data

- Values obtained from the AID3.2 counting were checked manually for each well.
- The number of spots obtained from each well was calculated as spots/ 10^6 splenocytes and averaged.
- Triplicates from incubation of different amounts of splenocytes were then averaged to obtain one value.
- The average number of spots obtained from each mouse from the incubation of the splenocytes with non pulsed A20 cells was subtracted to that obtained from stimulation with peptide pulsed A20.
- Final values per group of immunised mice and standard deviation was obtained by average of triplicate mice receiving the same immunisation.

2.4.5 ^{51}Cr release assay

2.4.5.1 Restimulation of splenocytes

^{51}Cr release assay was performed on splenocytes from immunised mice. These splenocytes were restimulated in vitro in a slightly different manner from § 2.3.6. Splenocytes for ^{51}Cr release assay were incubated together with irradiated splenocytes from non immunised mice, these have act as presenters of the peptide. Spleen cells from one non immunised mouse were prepared (§ 2.3.6) and resuspended in 10% (v/v) FCS in 1 x PBS in a 50ml Falcon tube. This was placed on ice within an X-ray machine and irradiated to 1500-3000 rad. Cells were then washed once with 10% (v/v) FCS in 1 x PBS, resuspended FGM and counted with trypan blue assay (§ 2.3.11). Irradiated cells were resuspended in FGM at $1-5 \times 10^5$ cells /ml. The responders splenocytes from immunised mice were resuspended at $2-3 \times 10^6$ cells/ml and 9ml placed in a 25cm² tissue culture flask to which 1ml of irradiated cells were added together with 10 μM of appropriate peptide. The flasks were incubated upright at 37°C, 5%CO₂ for 5 days.

2.4.5.2 Preparation of responder splenocytes

Cytotoxicity of the cells was measured by incubating restimulated spleen cells with antigen presenting cells pulsed with ^{51}Cr . Restimulated splenocytes were collected, washed twice with FGM by spinning at 321g (1300rpm) 5 minutes, counted and resuspended at 9×10^6 cells/ml in FGM.

2.4.5.3 Pulsing of target cells

Target P815 cells were used at 10^4 cells/well and effector:target ratios started at 90:1

Effector cells were resuspended in the appropriate volume. Two groups of target cells were formed, one to be pulsed with peptide at 10 μM and one to be left unpulsed. The required number of targets were suspended in 200 μl RPMI with/out peptide together with 100 μCi of ^{51}Cr and incubated at 37°C, 5%CO₂ for 1hour. Following this incubation, cells were washed three times in 20ml of warm RPMI and resuspended in RPMI 5%(v/v) FCS at a concentration of 10^5 /ml.

2.4.5.4 Assembly of ⁵¹Cr release assay

The test was assembled in 96-well round bottomed plates. The number of target cells was kept constant whereas responders were tested in serial dilutions. 100µl of targets were incubated with 100µl of responders in 1:3 serial dilutions. As positive control to measure the maximum ⁵¹Cr release from the target cells, these were incubated with 100µl of 2.5% (v/v) Triton X-100. Test was carried out both for targets that had been pulsed with peptide and those that had not, and each sample was tested in triplicate.

The plate containing the assembled assay was spun down 2 minutes 321g (1300 rpm) at room temperature to pellet the cells and then placed for 4 hours at 37°C, 5%CO₂. Following this incubation the plate was spun again 3 minutes 321g (1300 rpm) at room temperature and 25µl of the supernatant was transferred in 96 well sample counting plates (*Wallac*) and mixed with 175µl of SuperMix (*Wallac*). The plate was then sealed and mixed gently before being counted in a gamma scintillator counter (*Microbile Trilux*).

Percentage of killing was calculated as

$$\frac{(\text{c.p.m. in presence of responders} - \text{spontaneous } ^{51}\text{Cr release})}{(\text{Total } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release})} \times 100$$

2.4.6 Neutralisation assay

This was carried out to determine the presence of anti-HSV1 antibodies in the serum of immunised mice. The principle of this assay relies on the fact that if there are antibodies against the virus present in the serum, upon incubation with neat virus this will be neutralised, therefore resulting in the inability of the virus to infect monolayers of cells and form plaques.

5µl of serum from immunised mice (§ 2.5.6) were added to 45µl of serum free medium (DMEM) and these mixed in 96 well plates with 50µl of 200pfu/ml of 17⁺47⁻NP/vhsVP16⁻, or 17⁺47⁻GMCSFNP/vhsVP16⁻ in SFM. The mixture was left at 37°C, 5%CO₂ for 45 minutes before adding 200µl of SFM to each sample

and plating it in duplicate on monolayer cells for plaque assay (§ 2.3.9.2). The titres obtained from the duplicates were averaged. A positive control was always added to the samples consisting of incubation of virus without serum from immunised mice, this also gave a reading of the initial titre of the virus against which the other samples could be compared for possible reduction.

2.4.7 Tissue processing and immunostaining

10µm sections of lymph nodes snap frozen in Oct embedding medium (Cell Path) were cut on a cryostat (*Leica*) and placed on slides (§ 2.5.3)

Sections were dried and fixed in 4% paraformaldehyde (made in 1 x PBS) for 20 minutes at room temperature. Paraformaldehyde was then removed and the sections washed twice in 1 x PBS before being permeabilised with 20µg/ml proteinase K in 1 x PBS at room temperature for 20 minutes. Following this permeabilisation the sections were blocked for 20 minutes at room temperature with blocking solution (1% (v/v) FCS, 10mM Triton X-100, 10mM NaCl. The blocking solution was then discarded and the anti-HSV1 antibody HRP conjugated (*DAKO*) was added diluted 1:100 in blocking buffer. This was left on the sections overnight at 4°C and then removed by washing the sections twice with abundant blocking buffer. The secondary antibody swine anti-rabbit HRP diluted 1:100 in blocking buffer was then added on the section for 1 hour and then washed with three washes with blocking buffer. Antibody stain was developed with 3,3 N-Diaminobenzidine (DAB) (*Sigma*). DAB was made up at 1mg/ml in H₂O and then further diluted 1:2 in 1 x PBS. 12µl of H₂O₂ were added per 50ml of solution and placed on the slides until staining appeared. The reaction was stopped by washing with 1 x PBS.

2.5 Animal procedures

All animal procedures were carried out according to Home Office regulations after having obtained Home Office approval an animal handling licence.

BALB/c female mice were used through out this project. 3-4 weeks old mice were used to obtain the bone marrow whereas 6-8 weeks old mice were used for immunisation purpose. All immunisation procedures were carried out on mice sedated under 5% Halothane

2.5.1 Injections in the footpad

6-8-week-old female BALB/c were inoculated via left footpad injection using an insulin needle. 10^6 pfu of neat virus or cells in HBSS were injected in a maximal volume of 25 μ l.

2.5.2 Injections in flank

Female BALB/c mice received subcutaneous inoculations in the left flank. These were carried out using varying volumes of immunogen but usually not exceeding 30 μ l.

2.5.3 Lymph nodes extraction

Mice were sacrificed and popliteal and inguinal lymph nodes were removed from the inoculated sides and when necessary from the controlateral side as well. These were placed in Oct and immediately snap frozen on dry ice and placed at -80°C until processed.

2.5.4 Spleen removal

At the appropriate time after immunisation the mice were sacrificed and the spleens removed. These were placed in HBSS and processed within the hour.

2.5.5 Blood collection by cardiac puncture

Mice were sedated using Halothane and blood was then collected by cardiac puncture mainly from the left ventricle, while the hart was still pumping, using an insulin syringe. The blood was collected in 1.5 ml tubes for extraction of serum.

2.5.6 Extraction of serum

Serum was extracted from blood collected by cardiac puncture by centrifuging the blood for at least 50minutes at room temperature at 20798.5g (14000rpm). Upon this treatment the cells precipitate leaving a supernatant of serum. Serum was collected from each sample in a fresh 1.5ml tube and stored at -20°C until used. If planning to do more than one experiment or to repeat an experiment, aliquots were prepared and stored at -20°C until used.

Chapter 3

Identification of an HSV-1 vector for transduction of dendritic cells

3.1 Introduction

Herpes simplex virus 1 (HSV-1) has so far been used as a vector mainly for gene delivery to the nervous system. However, as it also naturally infects DC with high efficiency (Coffin et al., 1998, Kruse et al., 2000, Mikloska et al., 2001, Salio et al., 1999), and with minimal replication, it can also be considered for use as a vector to deliver genes to DCs for the stimulation of specific immune responses. A successful viral vector should present properties such as efficient and specific infection of the target cell type, as well as limited toxicity and sufficient levels of expression of the delivered gene. In addition, with respect to DCs, it is important that the ability of DCs to stimulate an immune response is not compromised. DCs can naturally present antigens and stimulate an immune response against infecting viruses, including inactivated non replicative viruses (Banchereau et al., 2000, Curran et al., 2000). However, this is only possible if the infected cell maintains its capacity to mature and upregulate costimulatory molecules (eg. CD40, CD80, CD86) that are necessary for the interaction of DCs with T cells and for the migration of DC to lymph nodes.

Many viruses are able to infect DCs, often as an immune evasion mechanism. HSV (Salio et al., 1999), like Sindbis virus (SIN) (Gardner et al., 2000), and influenza virus (Engelmayer et al., 1999), preferably infect immature DCs. However, unlike SIN and influenza, which stimulate cell maturation, HSV infection of immature DCs has been demonstrated to prevent cell activation (Kruse et al., 2000, Salio et al., 1999). HSV1 infected DCs also fail to produce a number of cytokines in response to activating stimuli, thus reducing their ability to stimulate T cell proliferation. Thus, in order for HSV-1 to be considered as a gene delivery vector for DCs, the virus must be modified appropriately such that DCs retain the ability to undergo their natural maturation process.

HSV1 expresses various genes to evade the immune system. For example, US12 encodes a protein (ICP47) which blocks the MHCI loading pathway. ICP47 is a cytosolic protein that binds via its N-terminus to the cytosolic peptide-binding domain of the human transporter associated protein (TAP) complex (Ahn et al., 1996, Tomazin et al., 1996, Tomazin et al., 1998) which normally translocates

peptides across the ER membrane. Interference with peptide transport (a mechanism also used by CMV and EBV) results in empty MHCI molecules being retained within the ER and therefore an impaired presentation of peptides by MHCI. Interestingly, the effect of ICP47 has only been detected in human DC infection and not in mice (Tomazin et al., 1998). In addition, HSV-1 expresses glycoprotein C and gE/gI, which respectively block complement activation and bind IgG. Although all these factors are important in preventing DCs from triggering an immune response, none of these is responsible for the inhibition of DC maturation caused by infection of DCs with HSV.

The work presented in this thesis focuses on the role of the virus host shutoff (vhs) protein. Vhs is a 58kDa protein encoded by the UL41 open reading frame (Kwong et al., 1988). This enters the cell upon infection and non specifically degrades cellular and viral mRNAs, mainly to benefit the translation of viral mRNAs, which are produced in high abundance. It has previously been observed that potent anti-HSV immune responses are stimulated by vhs deleted HSV (Geiss et al., 2000, Keadle et al., 2002). In this thesis, the role that vhs plays in human and mouse DC maturation is investigated using both vhs⁺ and vhs⁻ replication competent and attenuated HSV-1.

The toxicity of viral vectors is often reduced by preventing replication. HSV-1 replication takes place accompanied by a cascade of gene expression. The immediate early (IE) genes (ICP0, ICP4, ICP22, ICP27, ICP47) are expressed first, followed by early (E) genes and finally late (L) genes. ICP4 and ICP27 are absolutely required for viral replication (DeLuca et al., 1985, Sacks et al., 1985) and deletion of either of these produces a replication incompetent virus. Replication incompetent HSV-1 has previously been used and shown to be effective for gene delivery to neurones (Coffin et al., 1996, Lilley et al., 2001). These are also now being investigated for DC transduction. The so called 1764 viruses (Coffin et al., 1996) used in this thesis have γ 34.5 (the neurovirulence factor) deleted, and the *in1814* mutation in VP16 (viral transactivator) which inserts an in-frame linker sequence (Ace et al., 1989) to prevent its transactivation

properties. ICP4, ICP27, ICP47 and vhs are additional deletions used in some of the viruses.

Less attenuated viruses in which ICP27 and ICP4 were not deleted were also used in the work presented. Viruses which have a VP16 mutated are still replication competent. VP16 is a structural HSV protein released into the cell upon infection. It then enters the nucleus with the viral genome and together with cellular factors transactivates the promoters of the HSV immediate early genes (Herr, 1998). As the protein is an essential structural component of the virion and can not be deleted, mutations are used which only affect its transactivating properties. The VP16 mutation used here is either the V422 mutation (Lam et al., 1996, Smiley & Duncan, 1997), or the *in1814* (Ace et al., 1989). The V422 mutation consists of the truncation of the C-terminal acidic activation domain by a chain termination mutation following codon 422. This mutation removes the majority of the C-terminal acidic transcriptional activation domain impairing viral replication. Previous studies have shown that the V422 mutation greatly reduces IE genes expression (Lilley et al., 2001, Mossman & Smiley, 1999, Smiley & Duncan, 1997).

The aim of this chapter is to take advantage of the ability of HSV1 to infect DCs and to develop a virus that is minimally toxic to the cells and which will allow infected DCs to mature and stimulate an immune response. An HSV1 vector with these properties might then be used for immunotherapy for a number of diseases.

All the experiments presented in this chapter were repeated at least three times although representative FACS profiles from only one experiment are presented.

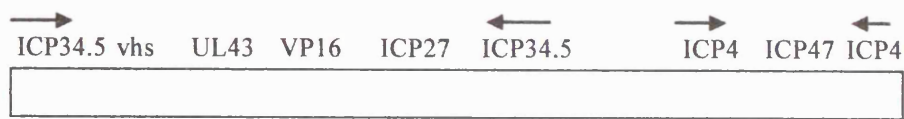
Viruses used in this chapter

This diagram indicates the progression of virus construction
Specific deletions and inactivations of each backbone are specified

X indicates inactivity of the gene either by deletion or inactivation
→ indicates the direction of gene expression

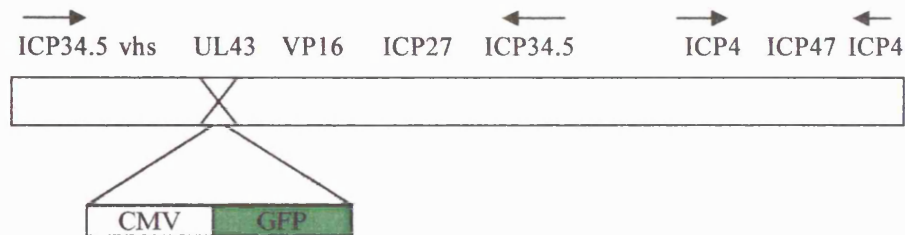
17⁺

Wild type HSV strain.



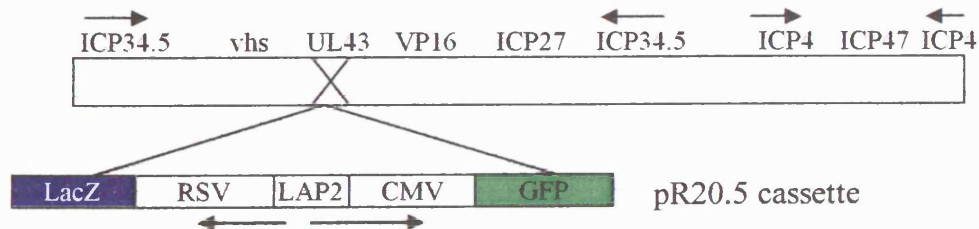
17⁺CMVGFP/UL43

UL43 gene inactivation by insertion of a CMVGFP cassette.



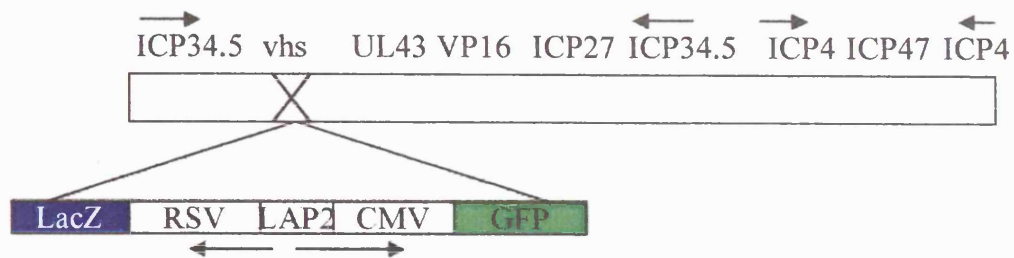
17⁺pR20.5/UL43

UL43 gene inactivated by insertion of the pR20.5 cassette
(Thomas, Lilley et al. 1999) The LAP2 region is kept because it
allows better expression of both promoters in neuronal cells



17⁺pR20.5/vhs

vhs gene disrupted by insertion of the pR20.5 cassette

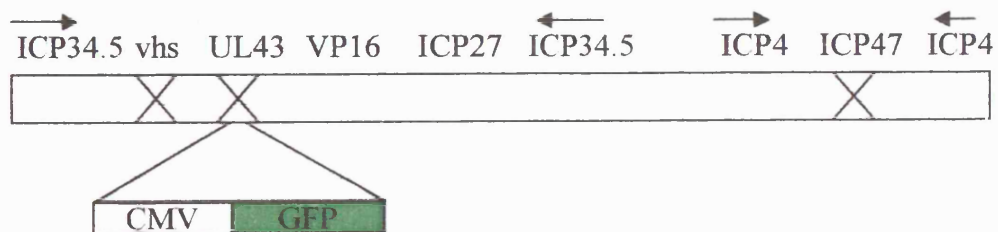


17⁺vhs⁻CMVGFP/UL43

vhs gene deleted

UL43 gene disrupted by insertion of a CMVGFP cassette

ICP47 gene deleted

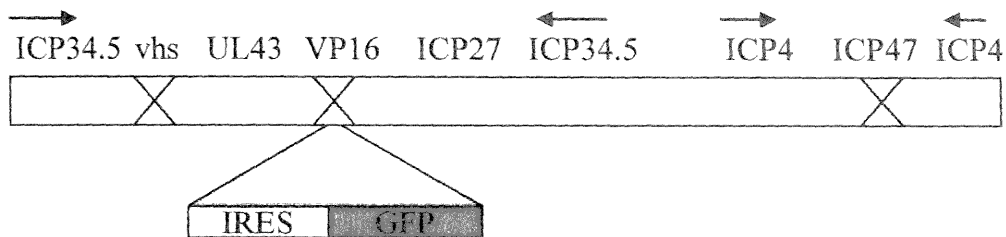


17⁺vhs⁻IRESGFP/VP16

vhs gene deleted

VP16 gene with V422 mutation and insertion of IRES GFP cassette

ICP47 gene deleted



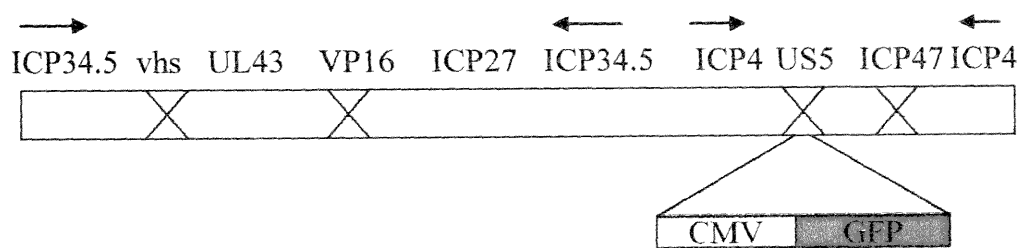
17⁺vhs⁻47⁻CMVGFP/US5 VP16⁻

vhs and ICP47 genes deleted

VP16 with V422 mutation

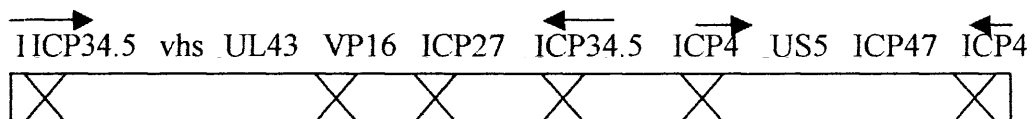
US5 inactivated by insertion of a CMVGFP cassette

ICP47 gene deleted



1764 27-4

ICP4, ICP27 and ICP34.5 genes deleted
in1814 mutation of VP16



1764 27-4 vhs

ICP4, ICP27, ICP34.5 and vhs genes deleted
in1814 mutation of VP16



3.2 Infection of human DC with replication competent HSV-1

3.2.1 Efficiency of infection of DC with 17⁺ and 17⁺pR20.5/UL43

The first requirement for a viral vector is to infect target cells, efficiently while avoiding toxicity. HSV 1 has previously been shown to infect DCs efficiently (Coffin et al., 1998, Kruse et al., 2000, Mikloska et al., 2001, Salio et al., 1999). Here, wild type 17⁺ and a 17⁺ viruses expressing the marker gene GFP inserted in the non-essential gene UL43 (Thomas et al., 1999) were tested for infection of human DCs. Two different GFP expressing viruses were used: one in which GFP driven by CMV is inserted into the UL43 gene, and the second one in which the GFP gene is expressed from the pR20.5 cassette inserted into UL43. The percentage of infected cells at MOI 1 was then determined by FACS, looking at the percentage of anti-HSV stained cells as well as at the percentage of GFP fluorescent cells where possible (17⁺ CMVGFP/UL43 and 17⁺pR20.5/UL43). It appeared that wild type HSV-1 is able to infect up to ~80% of human DCs at an MOI of 1 (figure 3.1) and that this percentage is not affected by the presence of a marker gene. In addition, it was noticed that, staining for anti-HSV gives slightly higher numbers of infected cells than is evident by measuring GFP fluorescence.

Infection of human DCs with 17⁺ at an MOI 1 is non productive (figure 3.2). Although some infectious viral particles appeared to be produced between 20hr and 24hr post infection, the total amount of viral particles revealed never reaches the initial input levels and continuously decreases after 24hr post-infection.

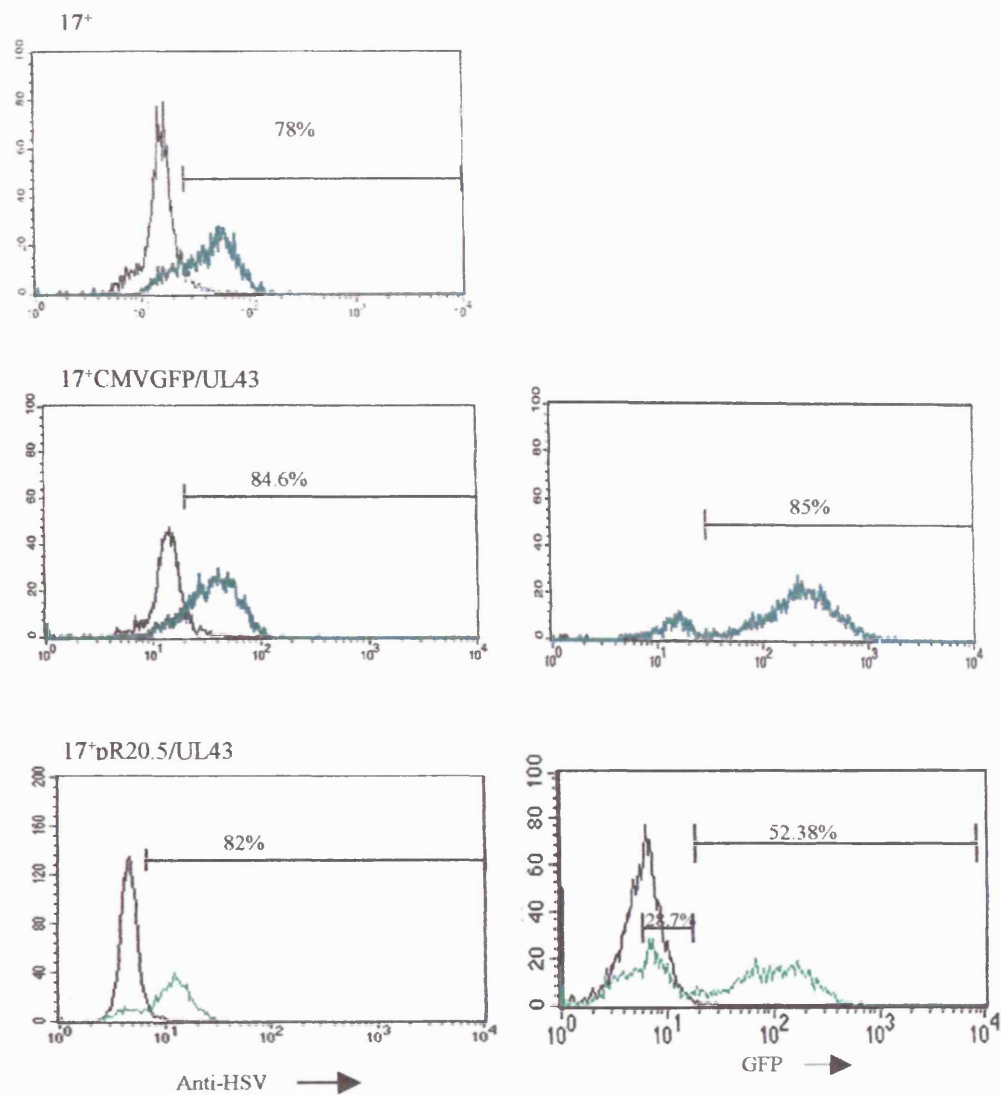


Figure 3.1: Infection of DC with HSV-1

Percentage of infected cells as determined by FACS analysis of anti-HSV stained cells and by GFP expression. Infected cells (green lines) are compared to non infected cells (black lines)

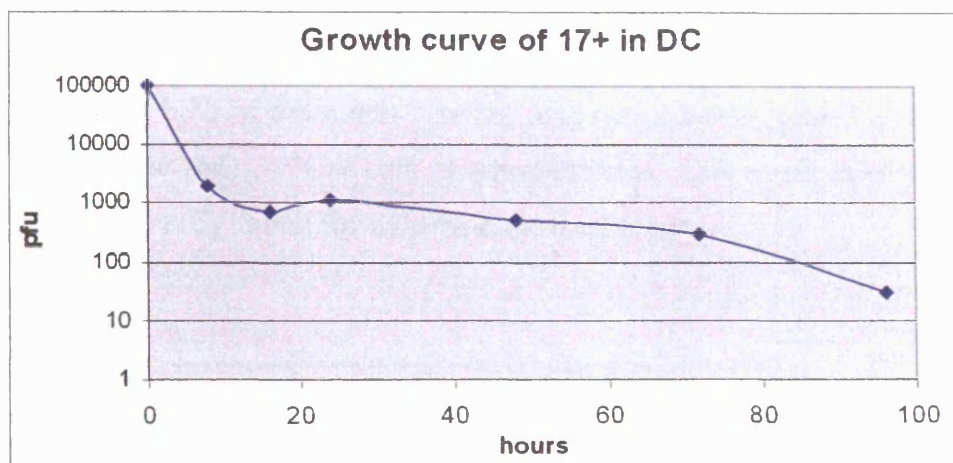


Figure 3.2: Growth characteristics of HSV-1 in DC

Growth curve of HSV-1 strain 17⁺ in human DCs infected at an MOI of 1. The yield was determined by titration on BHK cells at the indicated time points.

0hr value corresponds to the amount of virus used to infect the cells, this was obtained by titration of the virus put on the cells. This is not the titre obtained from the supernatant immediately after putting on the cells.

3.2.2 Efficiency of infection of DC with 17⁺pR20.5/vhs

HSV-1 infection is known to interfere with DC maturation (Kruse et al., 2000, Salio et al., 1999). In order to determine the possibility that vhs may play a role in blocking DC maturation, 17⁺ viruses differing only for the presence or absence of functional vhs were tested. Inactivating vhs by insertion of a pR20.5 expression cassette still gave ~80% infection, implying that removing vhs had no effect on the ability of the virus to infect DC (figure3.3). As previously noticed, looking at GFP fluorescence, three populations were found, two of which can be considered positive, and taking both of them into account (as previously suggested as being the most accurate method), 76% of cells resulted infected. This result relates well with the ~80% previously found for wild type virus infection.

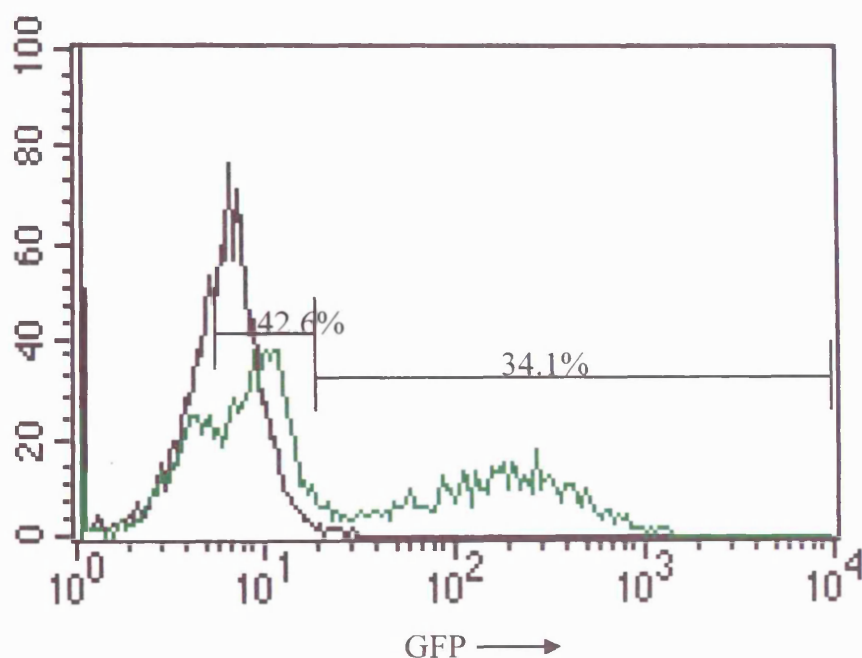


Figure 3.3: Infection of DC with vhs-HSV

Percentage of cells infected with 17⁺pR20.5/vhs at MOI1 according to GFP fluorescence (green). Negative control is non infected cells (black).

3.2.3 Effect of 17⁺pR20.5/vhs on DC maturation

The role that vhs plays on DC maturation was investigated by comparing the level of expression of cell surface markers (CD40, CD80, CD86) on cells that had been infected with 17⁺ or 17⁺pR20.5/vhs at an MOI 1 and then stimulated with LPS.

As expected, infection with 17⁺ prevented DC maturation (figure 3.4 and 3.5). Infection of DC with 17⁺ resulted in similar marker expression levels as mock infected cells, indicating that the cells remained immature. Infection with 17⁺pR20.5/vhs, on the other hand, clearly improved the ability of the cells to undergo maturation (figure 3.4 and 3.6). The profiles in this case showed a second population corresponding to mature cells. CD40 was upregulated to a greater extent. However, interestingly, CD86 showed two clear populations representing both the level of expression on immature and mature cells, indicating that although some maturation was occurring, inhibition had not been completely overcome. These experiments using wild type viruses with or without vhs clearly demonstrated that vhs alone plays a major role in the prevention of DC maturation. This work has subsequently been published in (Samady et al., 2003).

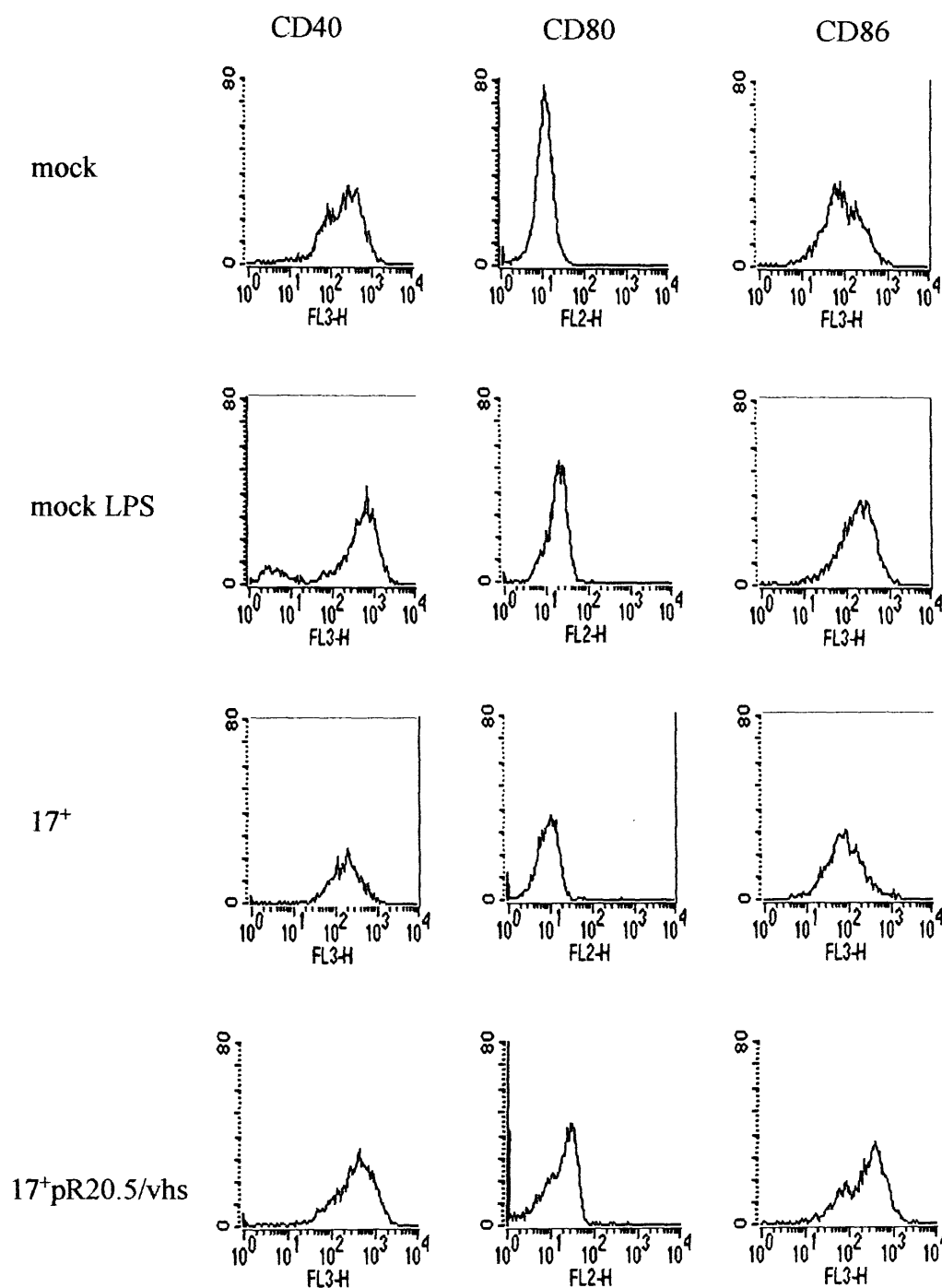


Figure 3.4: Effects of vhs on DC maturation

Histograms represent the level of expression of costimulatory molecules on human DC after 24hr infection at MOI1 with the specified viruses and LPS stimulation.

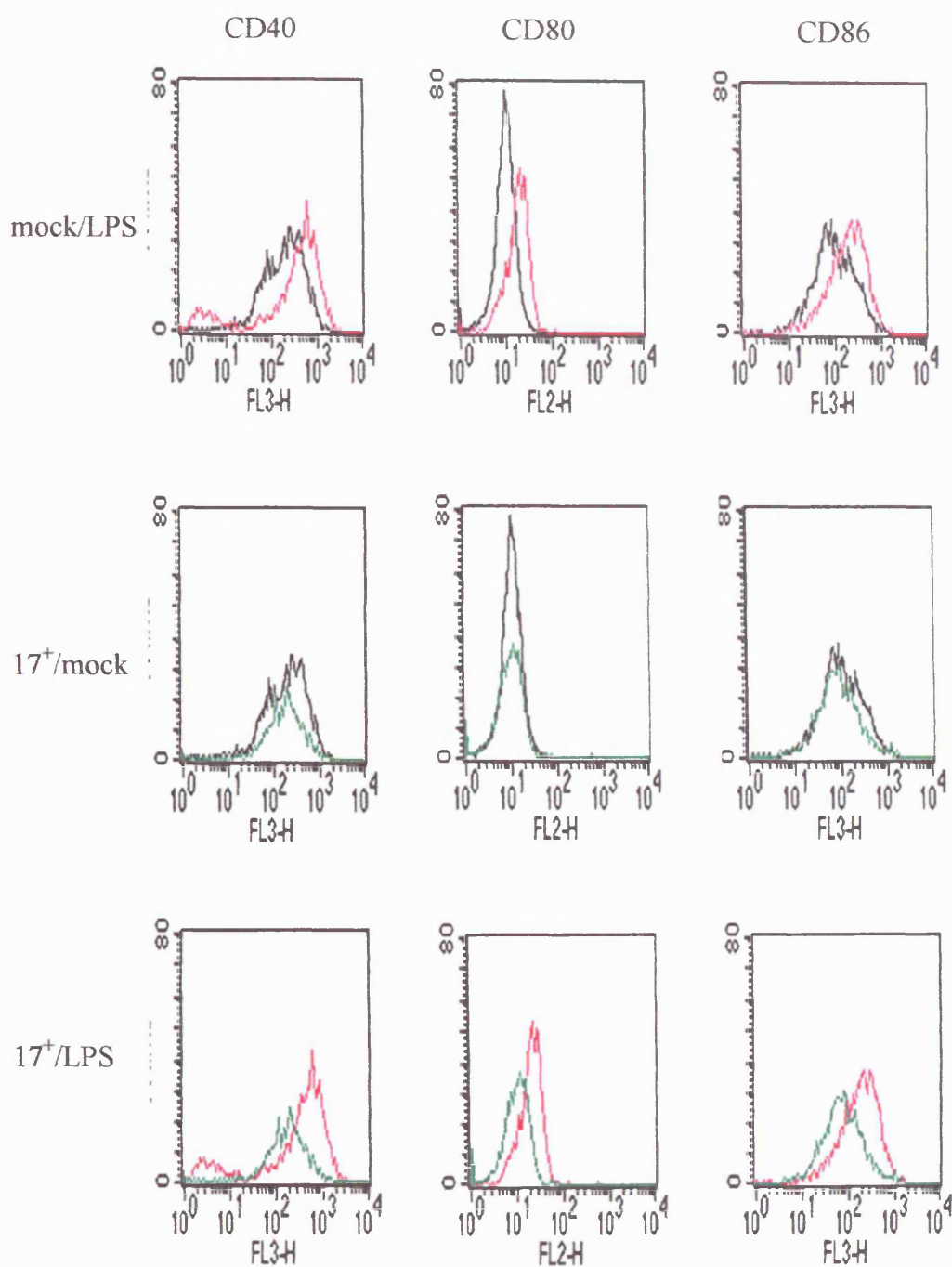


Figure 3.5: Inhibition of Dc maturation by HSV

Inhibitory effect of 17⁺ infection (green) on the level of expression of costimulatory molecules on human DC compared to the expression levels on non infected immature cells (black) and on non infected LPS mature cells (pink).

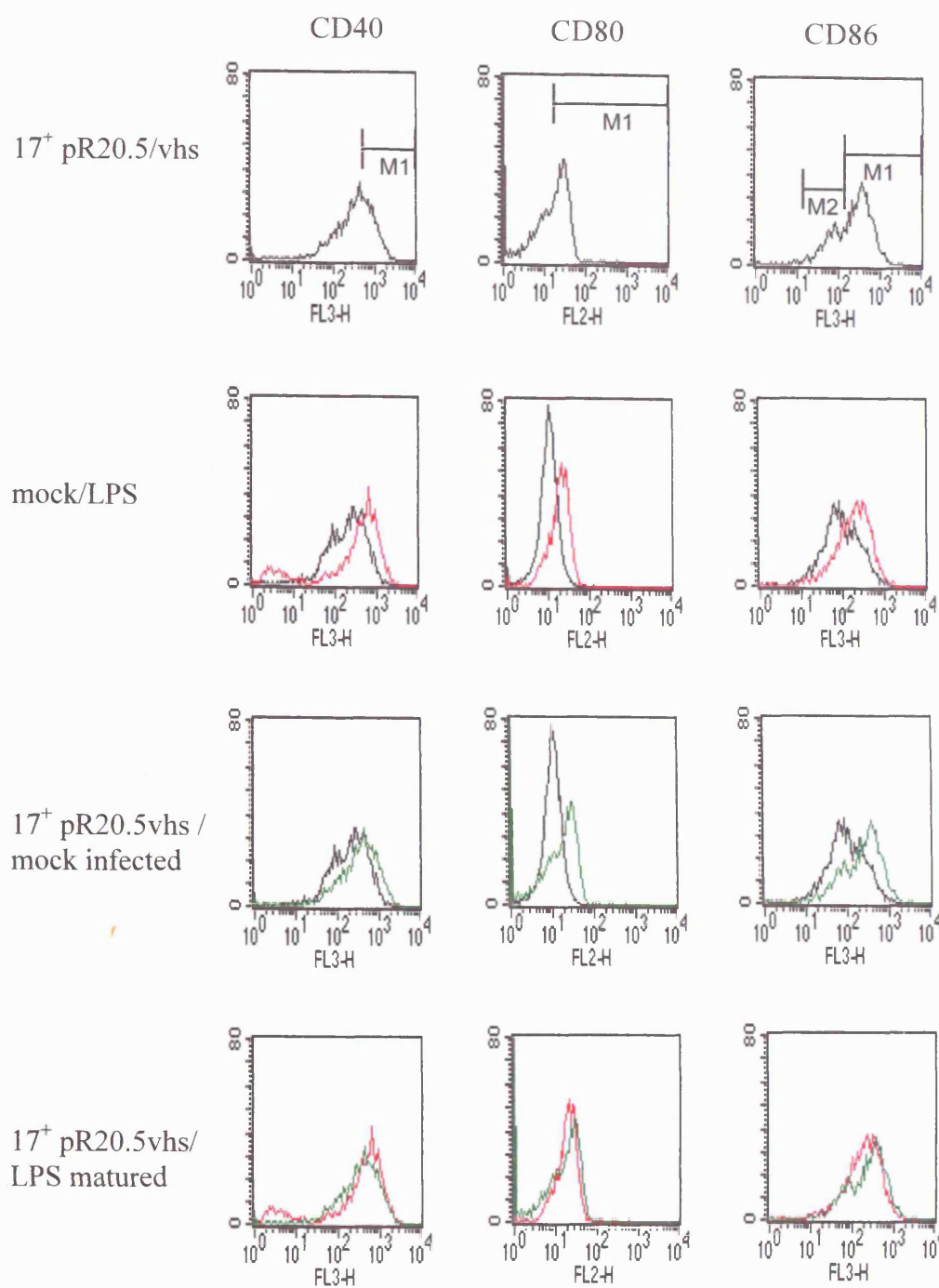


Figure 3.6: Effects of 17⁺pR20.5/vhs on DC maturation

Marker expression on human DCs stimulated to mature after infection at MOI1 with 17⁺pR20.5/vhs (green), and comparison with the expression on immature cells (black) and on LPS matured cells (pink).

3.3 Infection of human DC with replication incompetent HSV-1

3.3.1 Efficiency of infection of DC with 1764 27-4- and 1764 27-4-vhs-

Replication incompetent viruses have previously been shown in our laboratory to have a beneficial effect on cell viability (Samady et al., 2003) as compared to replication competent viruses. The effect of removing vhs from viruses of this type was therefore investigated further. Two 1764 replication incompetent viruses both lacking the immediate early genes ICP27, ICP4, as well as ICP34.5 and with the *in1814* mutation in VP16 but differing for the presence of the vhs gene were compared.

Although, as previously found with 17⁺ viruses, removal of vhs did not affect the efficiency of infection (figure 3.7), making the virus replication incompetent reduced its infectivity from ~80% (17⁺pR20.5/UL43) to ~57% (replication incompetent viruses), as determined by anti-HSV stain FACS analysis. The level of infection, though reduced, was still quite high as compared to other vector systems for DCs. It was therefore important to examine whether removing vhs from these vectors also had the beneficial effect previously seen using wild type HSV.

3.3.2 Effect of 1764 27-4- and 1764 27-4-vhs- on DC maturation

DCs were infected with the replication incompetent viruses with or without vhs, and the effect of vhs on cell surface maturation markers expression was assessed after LPS stimulation (figure 3.8-3.10). Here the CD40, CD80 and CD86 profiles from DC infected with the 1764 vhs⁺ virus (figure 3.9) more closely overlap those from non infected immature cells than those from mature cells. Thus, simply making the virus the replication incompetent virus does not prevent the block that HSV-1 causes to DC maturation.

Removing vhs from an already disabled virus, on the other hand, (figure 3.8 and 3.10) largely prevents the virus from interfering with the DCs normal maturation process. CD40 and CD86 in particular, showed complete overlap of the peaks from infected cells with those of mock infected LPS matured cells.

These results demonstrate that vhs is a key factor in preventing DC maturation upon infection with either replication competent or replication incompetent HSV1.

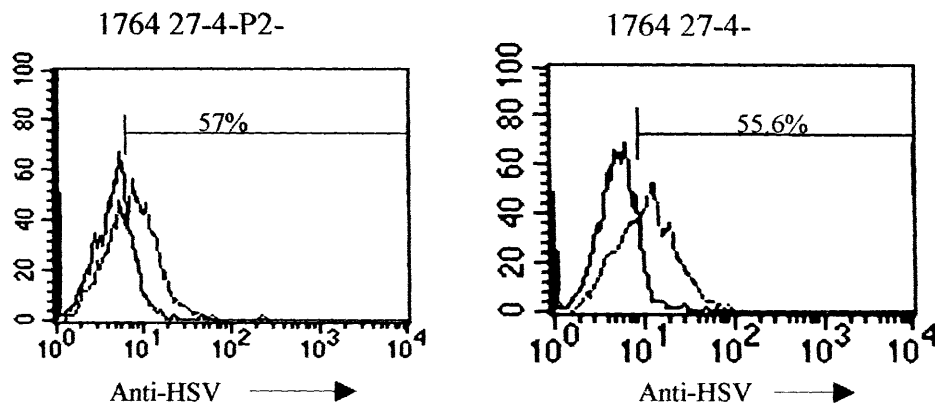


Figure 3.7: Infection of DC with replication incompetent HSV

Effect of removing vhs from replication incompetent viruses on the efficiency of infection. Percentage of infection was determined by FACS analysis of anti-HSV stained cells. Cells infected with the specified virus at an MOI of 1 for 24hr and stained for anti-HSV (green line) are compared to equally treated non infected cells (black line).

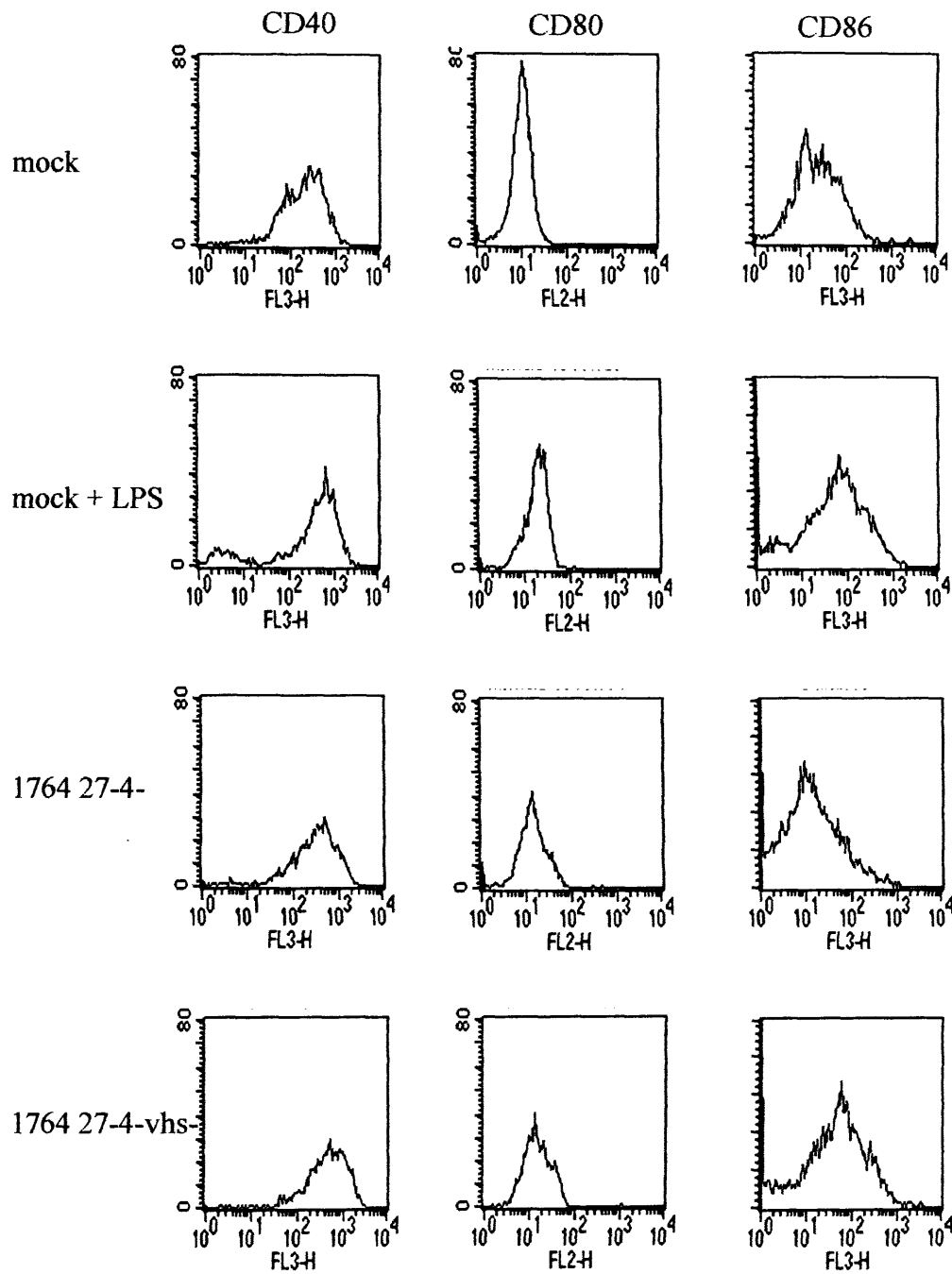


Figure 3.8: Effect of removing vhs from replication incompetent viruses

The level of expression of maturation markers on infected human DC. Cells were infected at MOI1 with the specified viruses and stimulated with LPS 24hr.

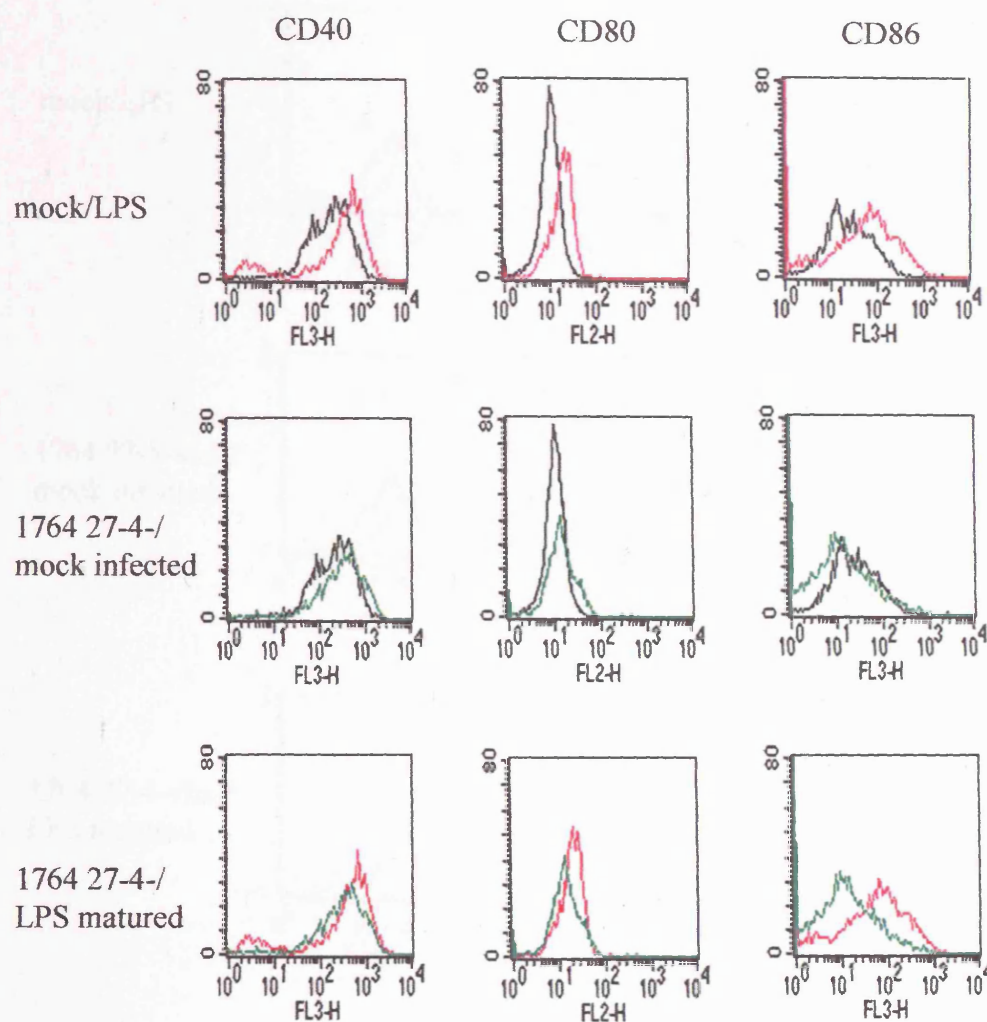


Figure 3.9: Effects of vhs^+ replication competent viruses on DC maturation

Level of expression of maturation markers on human DC after infection with 1764 27-4- and LPS stimulation 24hr (green), compared to the level of expression on immature mock infected cells (black) and on LPS matured mock infected cells (pink).

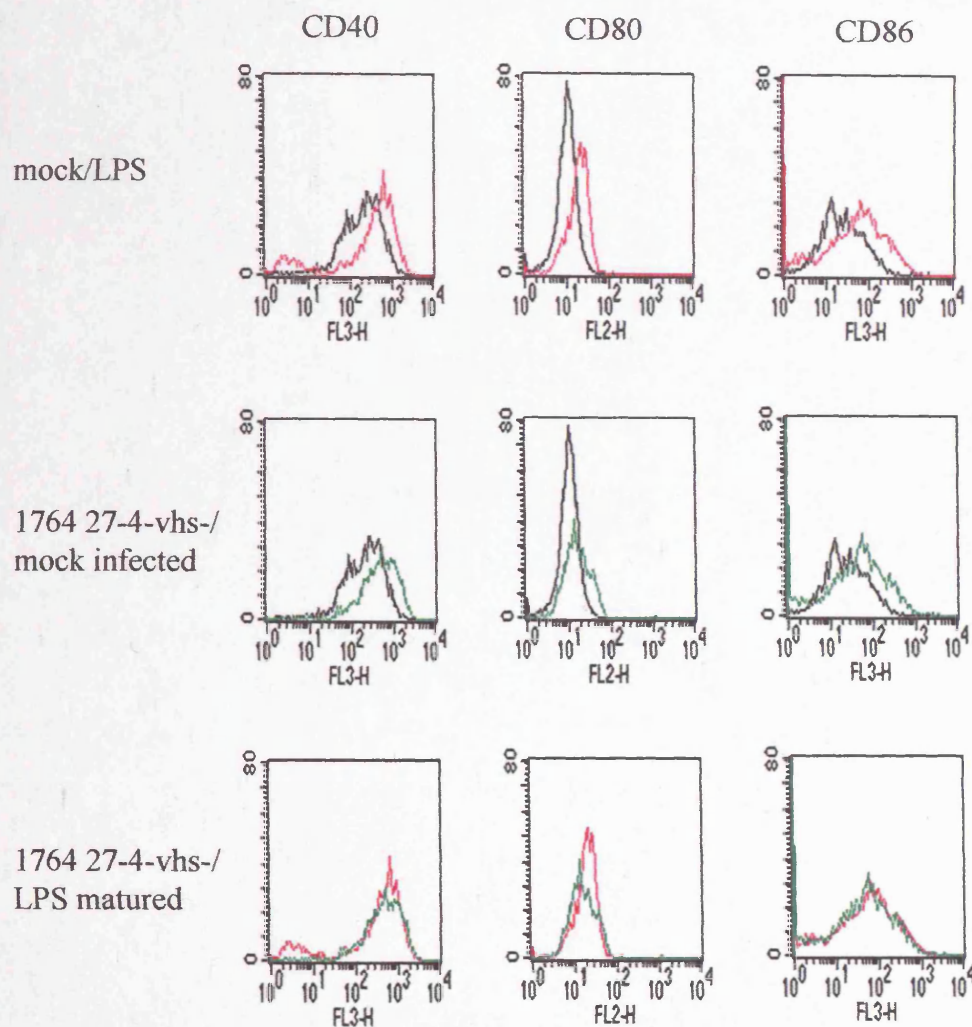


Figure 3.10: Effects of vhs- replication incompetent viruses on DC maturation

Level of expression of maturation markers on human DC after infection with 1764 27-4- vhs- and LPS stimulation 24hr (green), compared to the level of expression on immature mock infected cells (black) and on LPS matured mock infected cells (pink).

3.4 Optimisation of HSV-1 vectors for the transduction of mouse bone

marrow derived dendritic cells (BMDC)

3.4.1 Infection of mouse BMDC with 17⁺vhs⁻47⁻

To be able to conduct in vivo immunity studies in mice using HSV-1 vectors, as for human DC, it was important to identify a virus able to infect mouse bone marrow DCs efficiently without disrupting their ability to mature and to induce an immune response. The bone marrow contains precursors to a number of cell types, and different cells and DC subtypes develop depending on the cytokines present in the medium. BMDC cells cultured under GMCSF stimulation, lead to production of CD11c⁺ myeloid DCs able to induce IFN- γ mediated immune responses. In order to identify the minimally deleted virus needed, differently deleted replication competent viruses were tested for the efficiency of infection and toxicity in immature mouse BMDC.

The studies conducted on human DCs demonstrated that disruption of the vhs gene is an important factor in allowing maturation of infected cells. Equally, removing vhs did not make a difference to the ability of the virus to infect mouse BMDC (figure 3.11). As ICP47 impairs MHCI peptide loading, 17⁺vhs⁻47⁻ might provide an ideal virus backbone to stimulate immune responses. To allow easy detection of infection, GFP driven by a CMV promoter was inserted in UL43 (17⁺vhs⁻47⁻CMVGFP/UL43). Deletion of vhs has previously been shown not to change the virus ability to infect DCs. Similarly, removing the US12 (ICP47) gene should not influence infection but should increase antigen presentation on MHCI molecules.

Cells were infected with 17⁺ pR20.5/vhs or with 17⁺vhs⁻47⁻ and the percentage of infected cells was then determined by FACS (percentage of green fluorescent cells) (figure 3.11). Around 55% of cells became infected when ICP47 was present and ~ 60% when the gene was removed. ICP47 deletion therefore does not appear to compromise the ability of the virus to infect DCs. In addition, these results suggested that the virus infects mouse DC with lower efficiency than human DCs where ~80% of cells were usually found to be infected.

The specificity of the cell type infected was tested using $17^+vhs^-47^-$. This resulted in ~60% of the cells within the preparation being infected (GFP⁺), practically all of which (56%) expressed CD11c⁺ (figure 3.11). The CD11c⁻ infected cells (5.5%) were thought to probably still be in the precursor stage. Non infected cells (~22%) can be accounted for by the fact that the method of culture did not include any depletion or enrichment stage, and these cells are therefore probably other types of monocytes. If only CD11c⁺ cells are taken into account (making the population comparable to that obtained for human DCs), ~79% of cells were infected. It can therefore be concluded that the virus infects mouse DCs as efficiently as human DCs but that the lower percentages obtained were probably only due to differences in the purity of the initial population. As $17^+vhs^-47^-$ virus infected well and specifically, this was therefore characterised further in mouse BMDC infection.

3.4.2 Characterisation of $17^+vhs^-47^-$ infection of BMDC

The $17^+vhs^-47^-$ virus was found to efficiently infect BMDCs to an MOI as low as 0.2. Although the percentage of infected cells remained similar within the MOI range 0.2-1 (figure 3.12), the GFP intensity increased constantly, indicating the rise in the number of viral particles producing GFP per cell. This further demonstrated good infectivity for DCs. Because in the construction of a gene delivery vector strong expression of the recombinant gene is important, an MOI1 was used for subsequent experiments.

HSV-1 is not known to replicate within DC and both 17^+ and $17^+vhs^-47^-$, though being replication competent, were found to infect non productively (Figure 3.13 a,b). In addition, cells infected with the $17^+vhs^-47^-$ virus maintained relatively high viability (75%) 24hr post-infection as measured by the trypan blue exclusion assay (Figure 3.13c). $17^+vhs^-47^-$ therefore appeared to provide a promising vector for use with mouse BMDC.

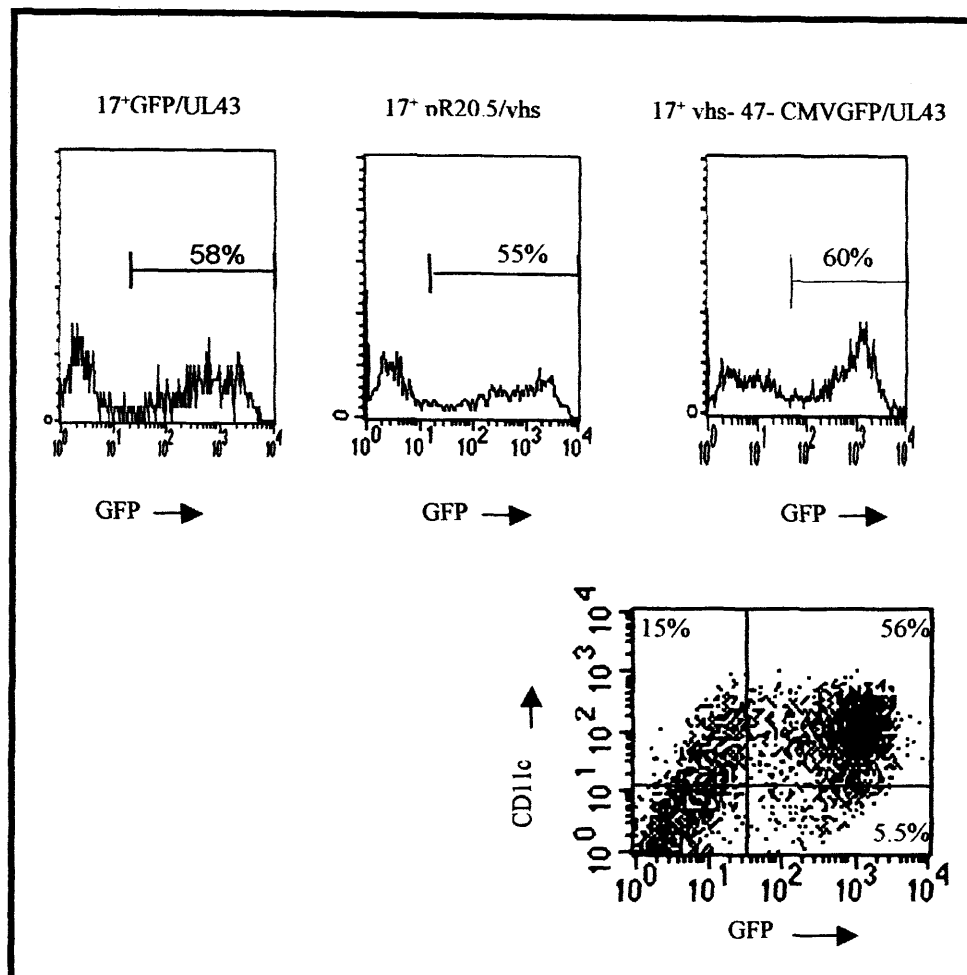


Figure 3.11: Effect of removing vhs and/or ICP47 on the ability of HSV-1 to infect BMDC.

Histograms showing the percentage of BMDC expressing GFP after infection at an MOI of 1 with 17^+ virus containing GFP (17^+ GFP/UL43), virus containing ICP47 but with vhs deleted (17^+ pR20.5/vhs) or virus where ICP47 and vhs have been removed (17^+ vhs⁻ 47-GFP/UL43). The marker indicates the level of fluorescence under which cells are considered non fluorescent, this was set according to the profile of non infected cells.

Dot plot of CD11c stained 17^+ vhs⁻47-GFP/UL43 infected BMDCs at MOI1, specific percentages indicate non CD11c infected cells and infected CD11c⁺

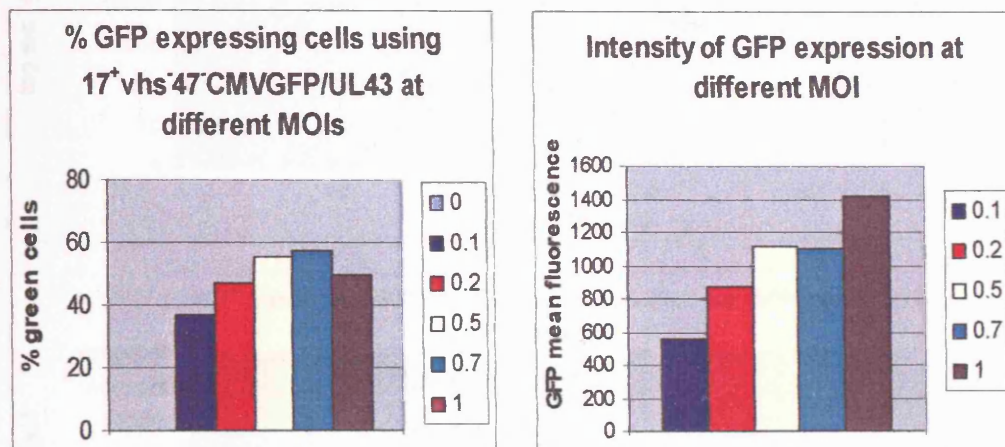


Figure 3.12: Efficiency of infection of 17⁺vhs-47-CMVGFP/UL43 at different MOIs

Values presented are the average of two or three samples.

(a) Percentage of BMDCs expressing GFP after infection at different MOIs with 17⁺vhs-47-GFP/UL43 for 24hr.

(b) Difference in GFP intensity expression upon BMDC infection at different MOI.

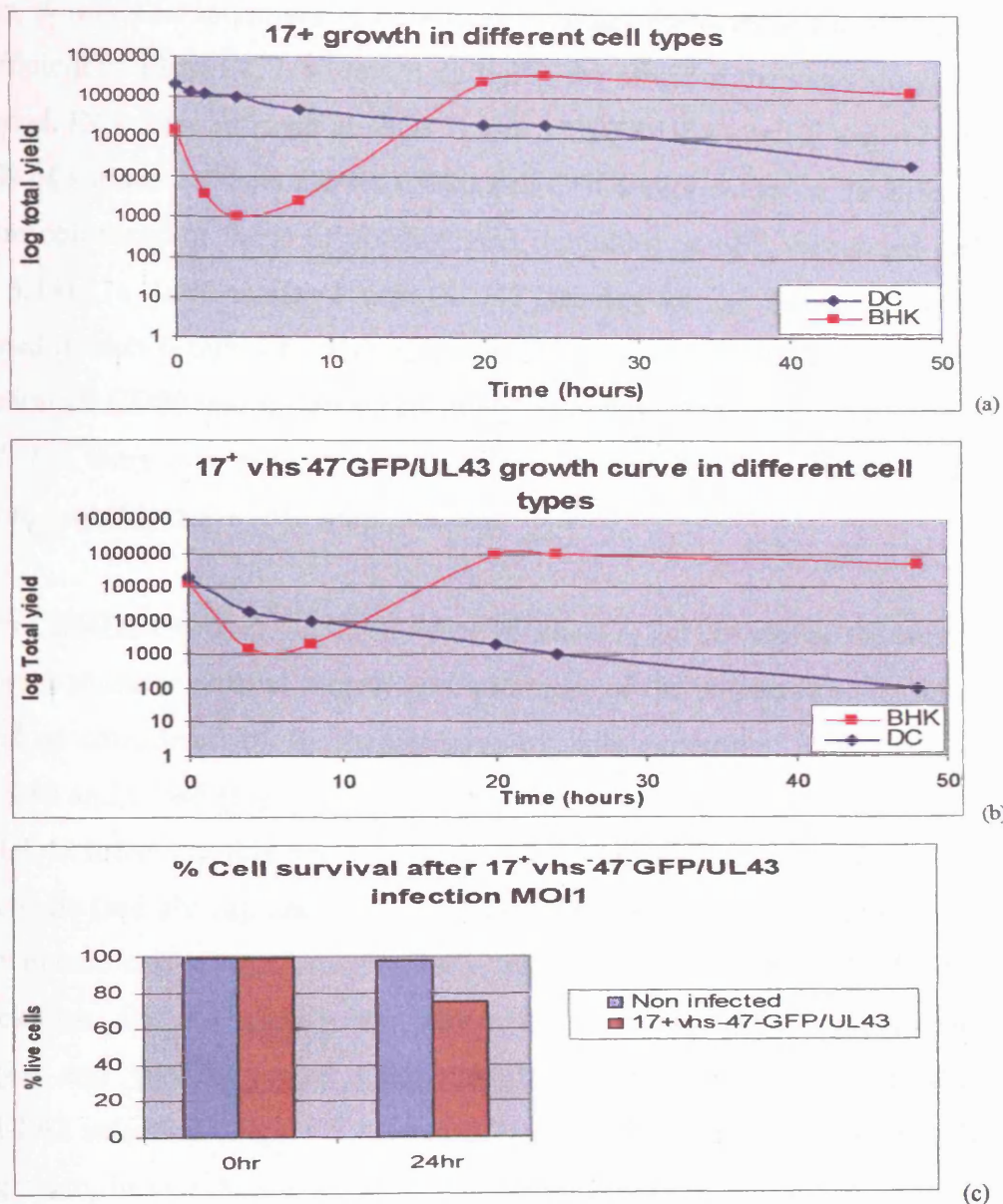


Figure 3.13: Assessment of the ability of HSV-1 vectors to replicate within mouse dendritic cells.

Growth curves of 17⁺ (a) and of 17⁺vhs-47-CMVGFP/UL43 (b) in BMDCs infected at an MOI of 1 compared to growth of the same viruses on Mam49 on which the virus is usually grown. The yield was determined by titration on Mam49 cells at the indicated time points. Toxicity of 17⁺vhs-47-GFP/UL43 on BMDC (c) measured by trypan blue exclusion assay, the value shown is the average of three counts of the same sample.

3.4.3 The effect of 17⁺ and 17⁺vhs⁻47⁻ infection on DC maturation

17⁺vhs⁻47⁻ was shown to efficiently infect BMDCs without apparent toxicity. However, it was also important to determine whether the deletions to this virus were sufficient to allow DC maturation. Initially, the effect of the virus alone was investigated. DCs were infected at MOI 1 with 17⁺GFP/UL43 or 17⁺ vhs⁻ 47⁻ and the levels of surface markers CD40, CD80 and CD86 expression on the infected cells were compared to those of non infected immature or LPS stimulated cells (Figure 3.14). In LPS matured cells CD40 seemed to be the most highly upregulated marker (~60% of cells), although 30% and 40% of cells also showed upregulation of CD80 and CD86 respectively. Looking at the immature cells, it appeared that there was between 5-8% of mature cells already present in the population, probably due to cell manipulation.

Infection of BMDCs with 17⁺ GFP/UL43 or 17⁺vhs-47- did not induce the cells to mature as the markers profiles largely overlaid those of the immature cells (figure 3.15), and as considered by the percentages of cells expressing high levels of CD40, CD80 and CD86 (Figure 3.14). ~10% of cells appeared to be mature after 17⁺GFP/UL43 infection, this being very similar to the percentage obtained from immature cells (see above), and showing that wild type virus infection does not appear to induce cell maturation. Similarly, removing vhs and ICP47 from the virus backbone did not change the effect of the virus on DC maturation, percentages and profiles were again similar to the ones obtained after 17⁺GFP/UL43 infection (Figure 3.14 and 3.16). These results were not entirely surprising since, in vivo too, it is not only antigen uptake or cell infection itself that induces cell maturation. The environment created around the infected cell is usually responsible for initiating maturation, which reaches completion upon arrival of the cell in the LN. The ability of infected cells to undergo maturation and effects of the deletions were therefore investigated after LPS stimulation.

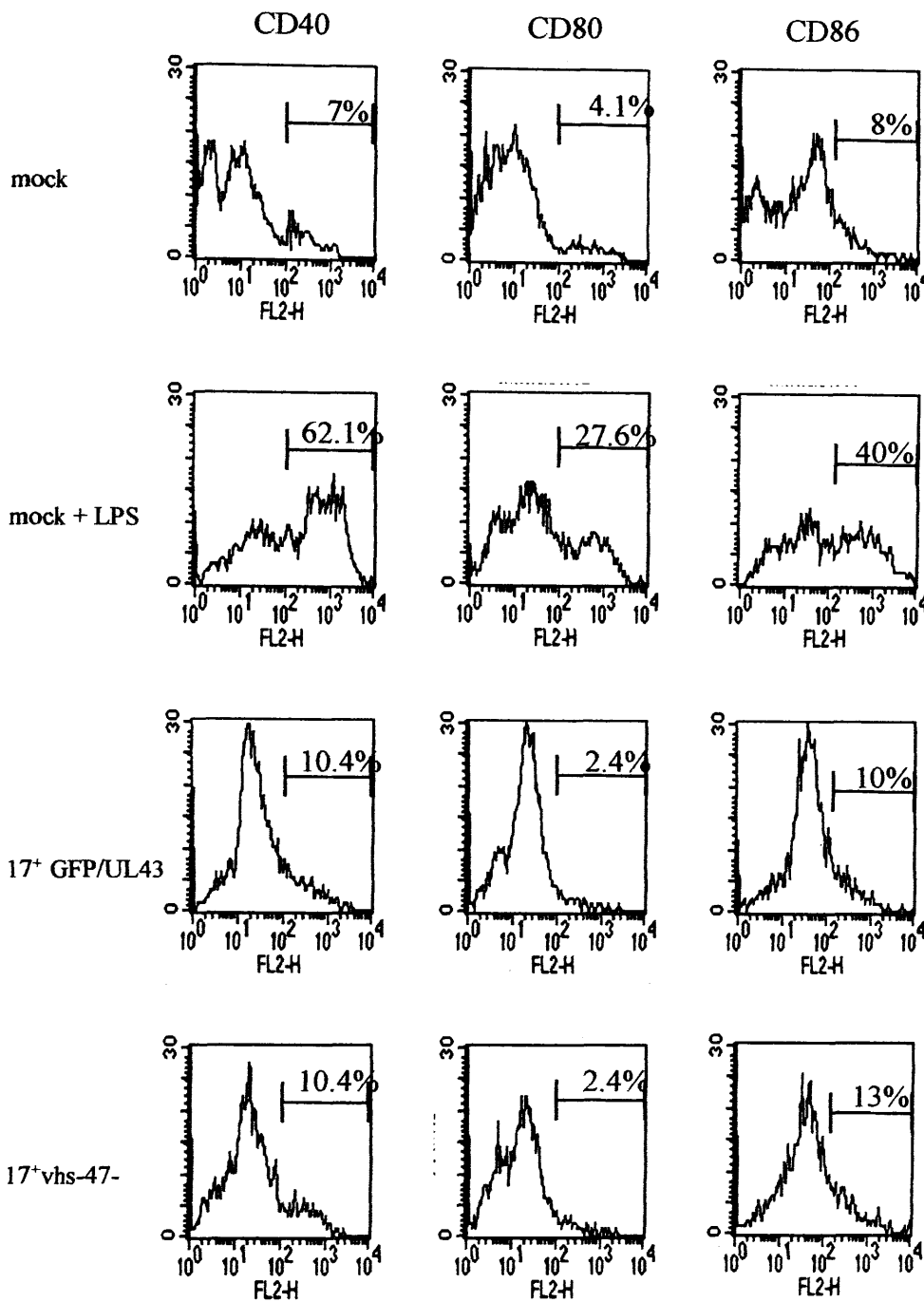


Figure 3.14: Effect of virus infection and deletion of vhs on BMDC surface marker expression.
 Percentages refer to the number of cells that have upregulated markers with same or similar intensity to mature cells.

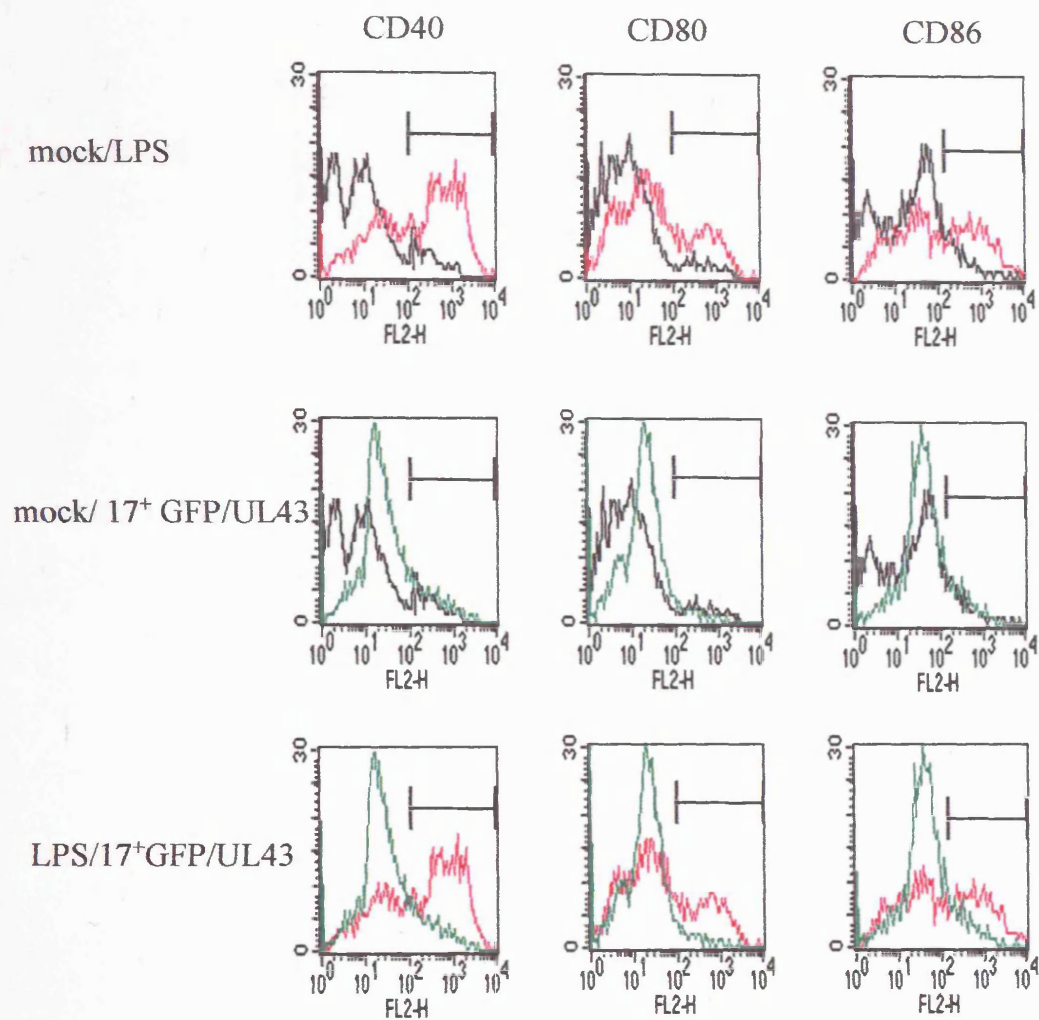


Figure 3.15: Effect of 17⁺GFP/UL43 infection of BMDC on the level of expression of maturation markers

Level of expression of maturation markers on BMDC after infection with wild type 17⁺GFP/UL43 (green), compared to the level of expression on immature mock infected cells (black) and on LPS matured mock infected cells (pink).

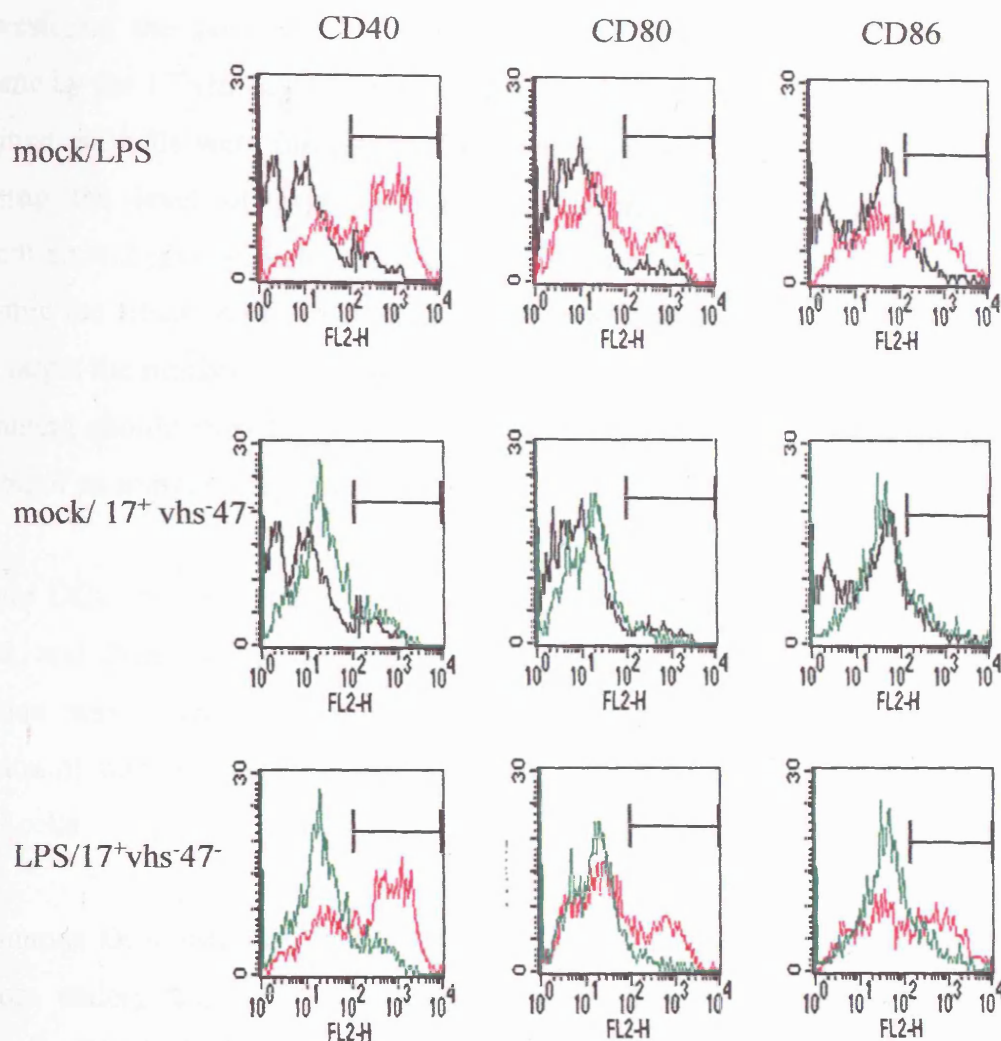


Figure 3.16: Effect of $17^+vhs^-47^-$ infection of BMDC on the level of expression of maturation markers

Level of expression of maturation markers on BMDC after infection with $17^+vhs^-47^-$ (green), compared to the level of expression on immature mock infected cells (black) and on LPS matured mock infected cells (pink).

3.4.3 Effect of 17⁺ and 17⁺vhs⁻47⁻ infection on the ability of DC to mature upon LPS stimulation

To investigate the possibility that the inhibitory effect of HSV-1 could be overcome by the 17⁺vhs-ICP47⁻ mutant, and to parallel as much as possible the in vivo situation, cells were infected and then induced to mature with LPS before measuring the level of expression of maturation markers expression. This treatment should give an indication of the role that vhs plays in DC maturation, and mimic the situation of direct virus injection in that, upon injection, the virus should target the immature DCs present around the site, this, plus the surrounding environment should then trigger cell maturation and travelling to the LN, with induction of an immune response.

Immature DCs were infected at MOI1 with 17⁺GFP/UL43, 17⁺vhs⁻47⁻ or mock infected, and then induced to mature by culturing in presence of LPS. Cell maturation was again assessed by FACS analysis, comparing the level of expression of surface markers CD40, CD80 and CD86 of infected cells with non infected cells.

As for human DCs, infection of BMDCs with 17⁺GFP/UL43 virus prevented the cells from undergoing maturation, showing FACS profiles and percentages of mature cells (Figure 3.17 and 3.18) practically identical to those of immature cells (~6%). Removing vhs and ICP47 from the virus, interestingly, improved the response of the infected cells to LPS (figure 3.17 and 3.19) showing both CD40 and CD86 upregulation in ~20% of the population. Not much upregulation was noticed in this experiment for CD80, though an improvement could still be seen. These percentages clearly showed an improvement compared to wild type virus infection and similarly, the profiles resembled more those of mature rather than immature cells (Figure 3.19). In addition it can be seen (figure 3.20) that the cells that show marker upregulation are not only those which are not infected, but most of the infected cells are able to mature.

Overall, as previously found for human cells, removing vhs seemed to have a beneficial effect on the ability of infected DC to undergo maturation, although

complete identity of profiles of matured infected and non infected cells was not yet achieved suggesting further virus attenuation might be required.

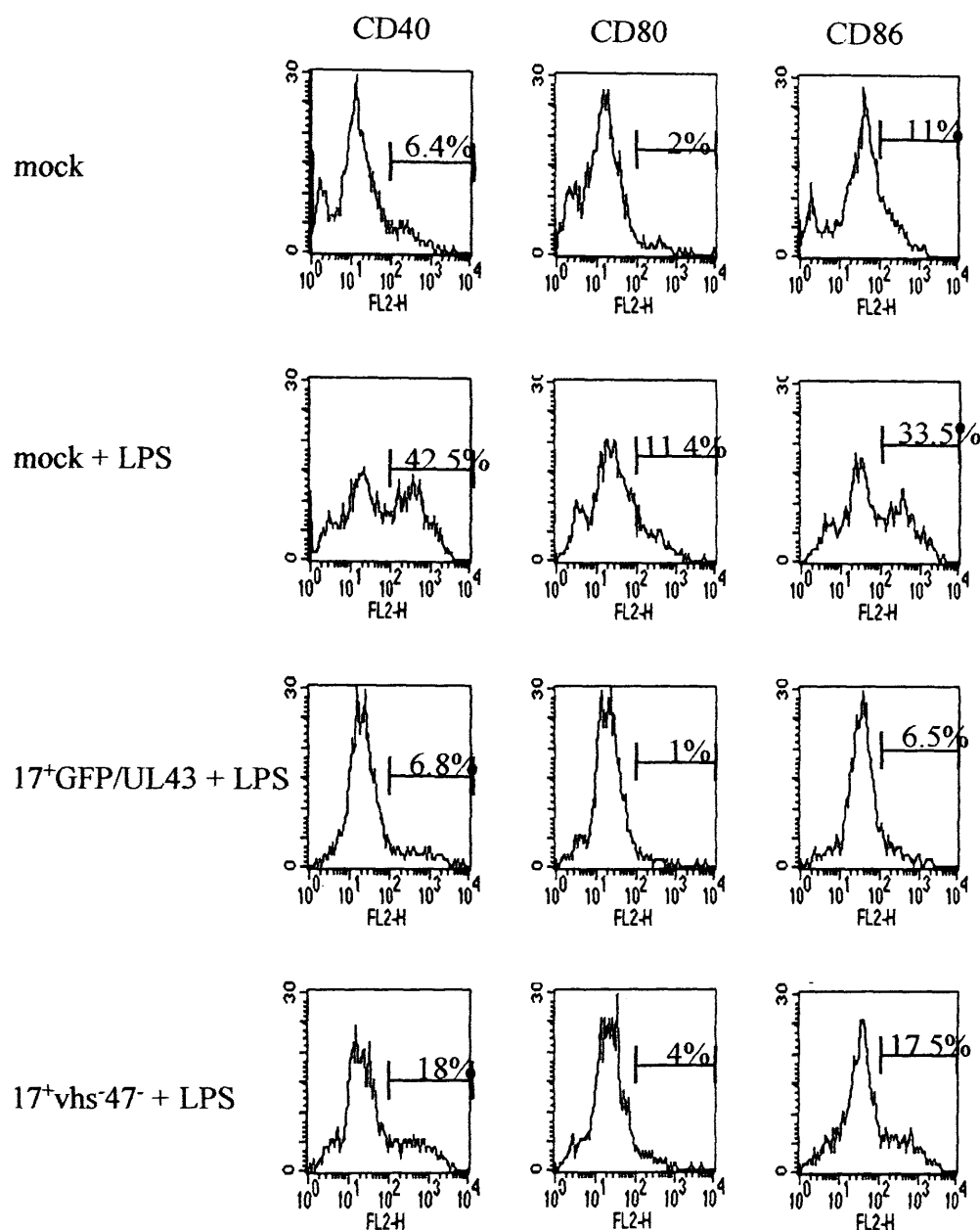


Figure 3.17: Level of expression of costimulatory molecules on infected BMDC stimulated with LPS

Expression of costimulatory molecules on BMDC infected at MOI1 with the specified viruses and stimulated to mature with LPS. The effect of removing vhs can be specified looking at the percentages which indicate the amount of mature cells present in the population after the different treatments.

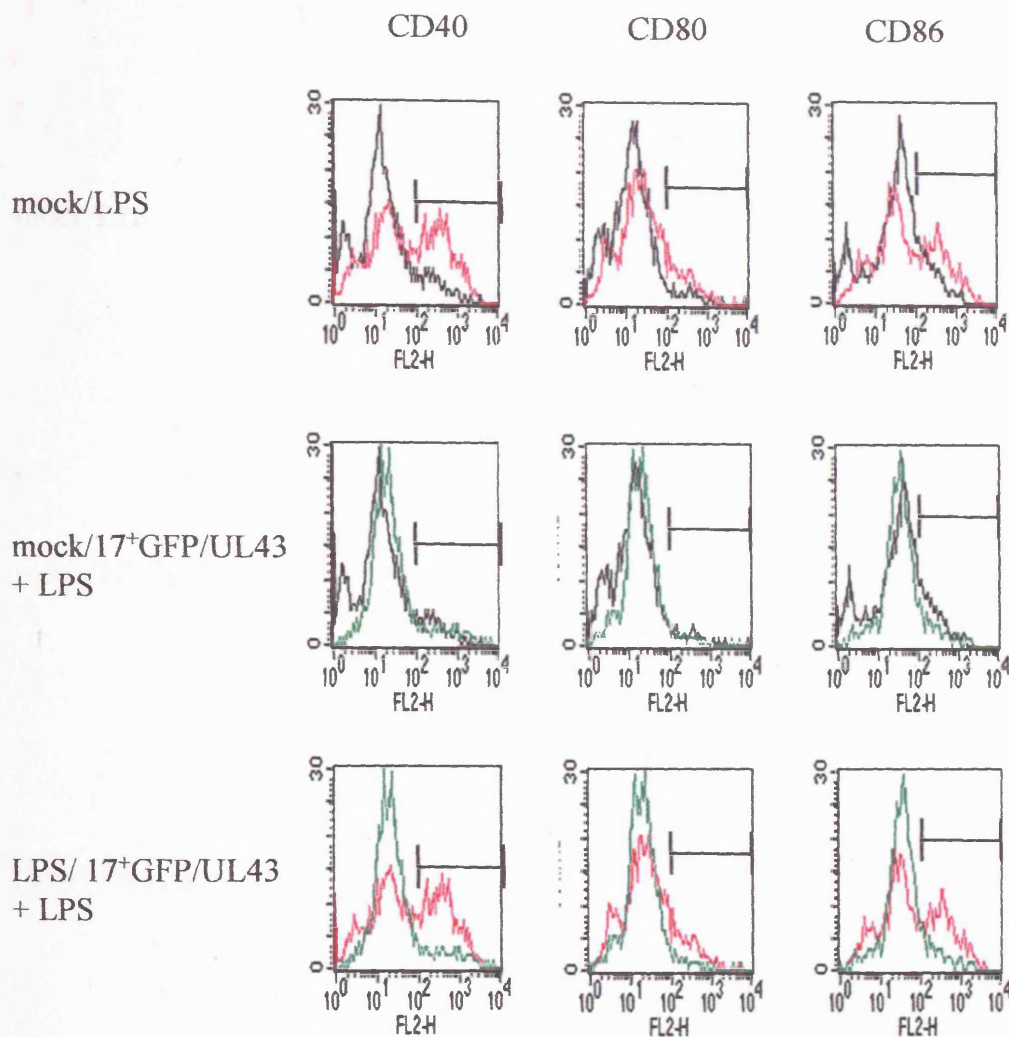


Figure 3.18: Effect of 17⁺GFP/UL43 infection on BMDC maturation
 Effect of wild type 17⁺GFP/UL43 infection on BMDC ability to undergo maturation upon LPS stimulation. Level of markers expression on BMDC infected at MOI1 and LPS stimulated (green) compared to level of maturation markers expression on immature cells (black) and on mature cells (pink).

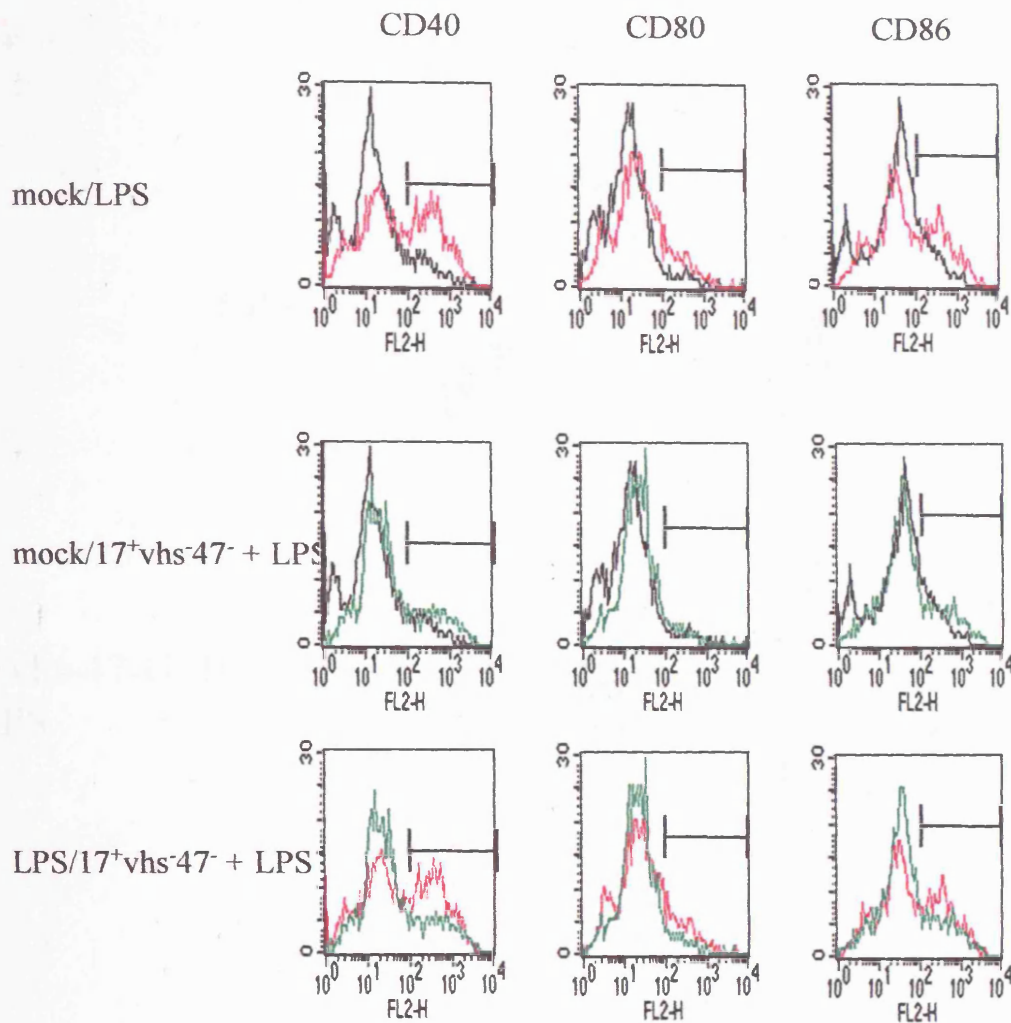


Figure 3.19: Effect of 17⁺vhs⁻47⁻ infection on BMDC maturation

Level of costimulatory molecules expression on BMDC infected at MOI1 with 17⁺vhs⁻47⁻ and stimulated with LPS (green) compared to immature cells (black) and to mature cell (pink) levels.

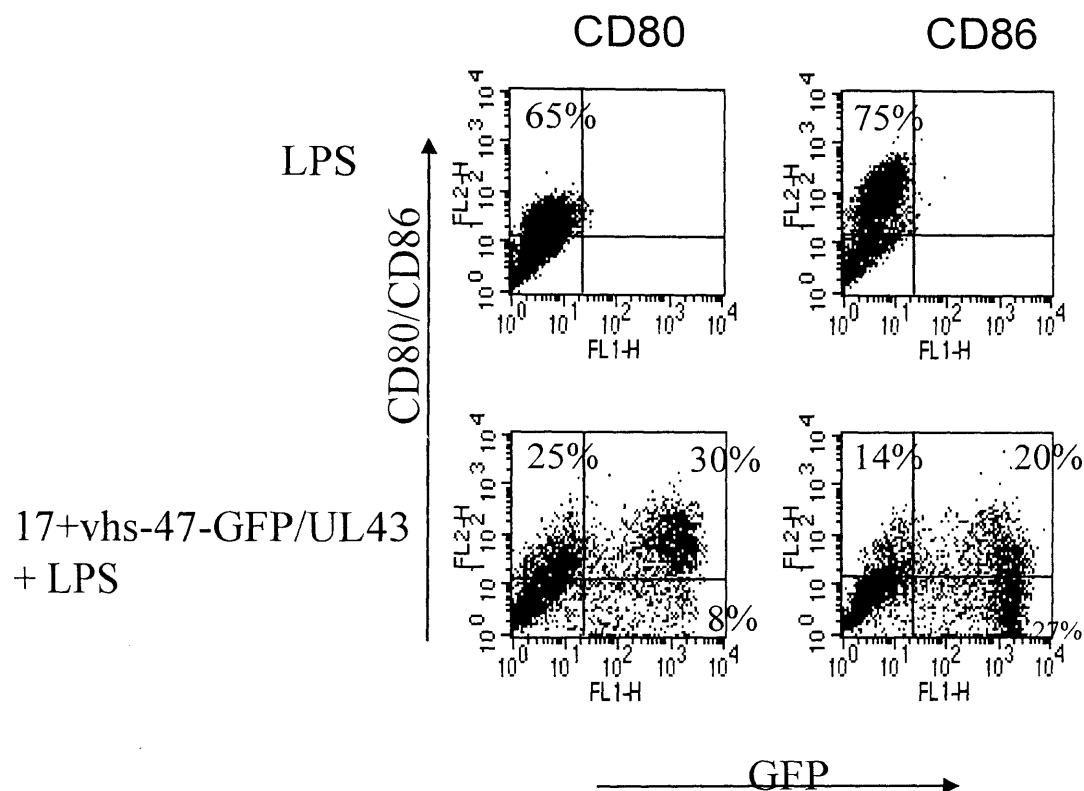


Figure 3.20: Expression of CD80 and CD86 in infected cells.

Dot plots showing simultaneous expression of GFP (FL1) and CD80 and CD86 markers (FL2) on non infected mature mouse BMDC (LPS) or BMDS infected with 17+vhs-47-GFP/UL43 and stimulated to mature with LPS.

3.5 Infection of mouse BMDC with 17⁺vhs⁻47⁻VP16⁻

3.5.1 Infection of BMDC with 17⁺vhs⁻47⁻ VP16⁻

In human DC it was found that better cell maturation was achieved after infection with replication incompetent viruses. Therefore, further attenuated backbone, though replication competent was tested on mouse BMDCs. This virus was mutated for VP16 as well as vhs and ICP47. The mutation in VP16 present was the V422 mutation (Lam et al., 1996, Smiley & Duncan, 1997), different from the mutation present in 1764 viruses previously used as the transactivation domain is completely removed rather than only disrupted (Ace et al., 1989)(§3.1).

The virus used was 17⁺vhs⁻47⁻ IRESGFP/VP16⁻ in which GFP following an IRES sequence is inserted within the mutated VP16 gene. Infection of DCs with this virus made detection of infected cells very difficult since GFP was only very weakly expressed in DCs. Expression of GFP from this site was also weak in complementing cell lines (Mam49) (figure 3.21). Failure to detect green fluorescence, made it necessary to attempt to detect the level of infection by antibody staining for HSV, however, unlike for human cells, non-specific binding of the anti-HSV antibody prevented this. Thus, finally, a virus of the same backbone but with GFP expressed from a CMV promoter inserted in the US5 gene (encoding for the viral glycoprotein gJ) was used. 17⁺vhs⁻47⁻ CMVGFP/US5VP16⁻ had increased, though still relatively faint fluorescence (figure 3.22), suggesting that mutation of VP16 may affect gene expression, although a virus with GFP expression from UL43 as for the previous viruses was not constructed to allow a definitive conclusion to be drawn.

Using 17⁺vhs⁻47⁻GFP/US5VP16⁻ it was possible to detect infected cells both by fluorescence (Figure 3.22a) and by FACS (figure 3.22b). Infection with 17⁺vhs⁻47⁻VP16⁻ led to ~80% infected cells, indicating that unlike in human cells where the 1764 viruses had reduced infectivity, the additional VP16 mutation does not influence BMDC infectivity in mice. Indeed, as previously ~60% of the total population of cells were infected, it appears that the attenuated vector infects mouse BMDC better than 17⁺vhs⁻47⁻, possibly due to reduced toxicity. Similarly to 17⁺vhs⁻47⁻ virus, this virus did not replicate in DCs (Figure 3.23a). Confirming

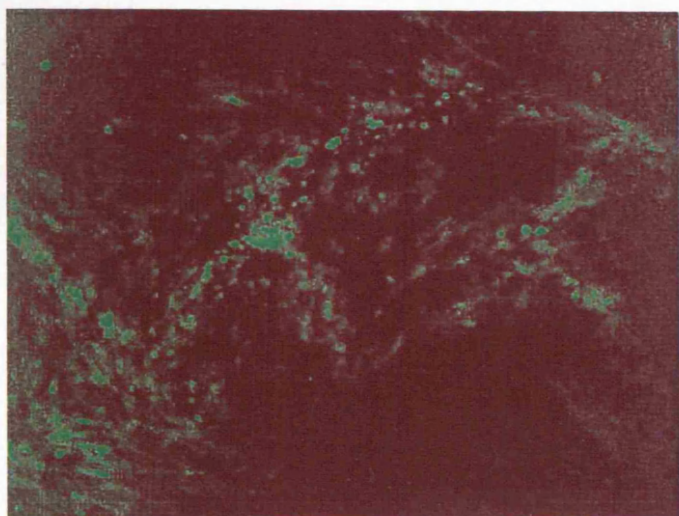


Figure 3.21: Weak GFP expression from 17⁺vhs-47-VP16⁻ infected Mam49 cells

40X magnification of plaque formed following 17⁺vhs-47-VP16⁻ virus infection of Mam49 cells.

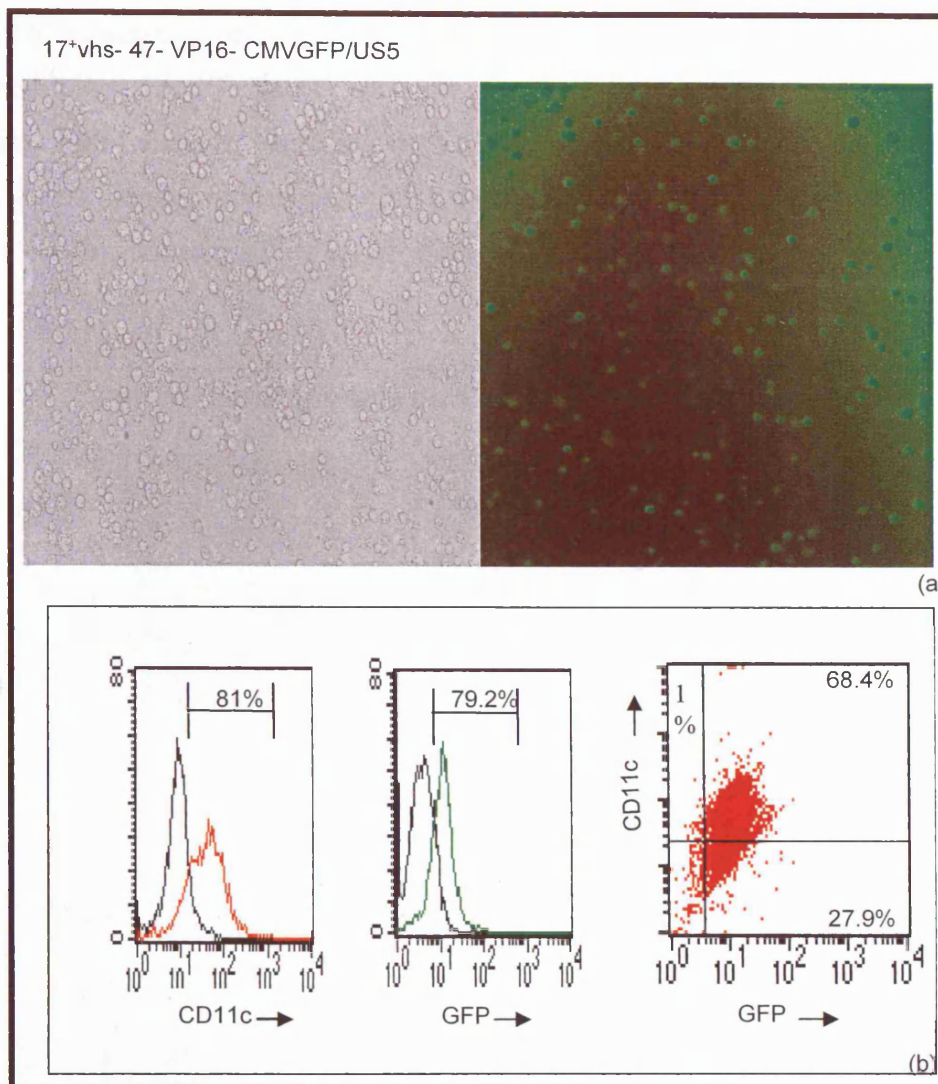


Figure 3.22: Infection of BMDC with 17⁺vhs-47⁻CMVGFP/US5 VP16⁻

(a): 40X BMDC infected MOI 1 with 17⁺vhs-47⁻CMVGFP/US5 VP16⁻. The two images represent the same field photographed under white light and fluorescent light to show simultaneously (i) total cells and (ii) infected cells expressing GFP. Comparison of (i) and (ii) indicates efficiency of virus infection.

(b): FACS data of BMDC infected at MOI 1 with 17⁺vhs-47⁻CMVGFP/US5 VP16⁻. Histograms show the percentage of CD11c⁺ cells in the population and the percentage of green, infected cells; the dot plot incorporates the two histograms indicating specificity of infection for CD11c⁺ cells.

the assumption that the VP16 mutated virus might be less toxic to BMDC (figure 3.23b), up to ~90% of cells remained viable until 48hr post infection with $17^+vhs^-47^-VP16^-$ as determined by trypan blue exclusion assay, compared to the ~75% obtained 24hr after $17^+vhs^-47^-$ infection (see fig 3.13). Overall, similarly to the situation with human DC infected with replication incompetent viruses (Samady et al., 2003), impairing viral replication by mutating the VP16 gene, resulted in a vector which retained good infectivity, but with reduced toxicity.

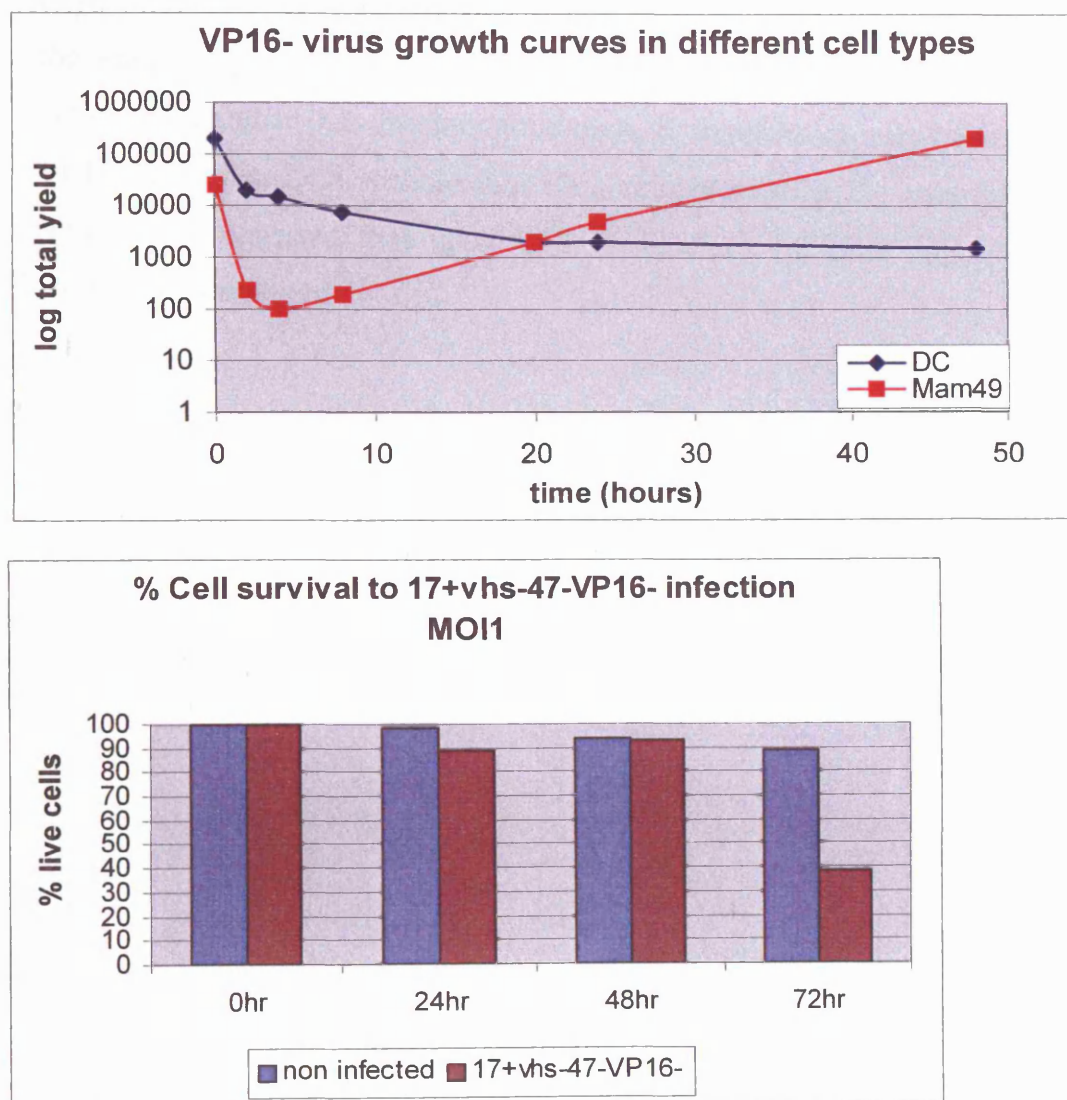


Figure 3.23: Characteristics of $17^+vhs^-47^-IRESGFP/VP16^-$ infection of BMDC

(a) Growth curve of $17^+vhs^-47^-IRESGFP/VP16^-$ in BMDC (blue) infected at an MOI of 1, compared to the growth curve in Mam49 (pink). Yield was determined at the time points indicated by titration on Mam49 cells.

(b) Toxicity of $17^+vhs^-47^-IRESGFP/VP16^-$ in BMDC as determined by trypan blue assay, values used are the average of three counts of same sample.

3.5.2 Effect of 17⁺vhs⁻47⁻VP16⁻ on DC maturation

17⁺vhs⁻47⁻VP16⁻ virus infected BMDC efficiently, did not replicate, and seemed minimally toxic. The effect of virus infection on cell maturation was therefore also investigated. For the following experiments, the 17⁺vhs⁻47⁻IRESGFP/VP16⁻ virus was used. Immature BMDC were infected at MOI1 and the expression profiles of the cell surface maturation markers were assessed by FACS 24hr post-infection. Interestingly, and differently from what previously found with other backbones, infection alone was able to stimulate a certain degree of maturation (figure 3.24 and 3.25). Percentages of upregulated markers (figure 3.24) as well as the markers profiles (Figure 3.25) clearly showed a greater similarity to the mature cells rather than the immature ones. It therefore appeared that 17⁺vhs⁻47⁻VP16⁻ can infect DCs without inhibiting activation, as is the case for wild type HSV. This suggested that upon LPS stimulation, the cells might be able to become fully matured.

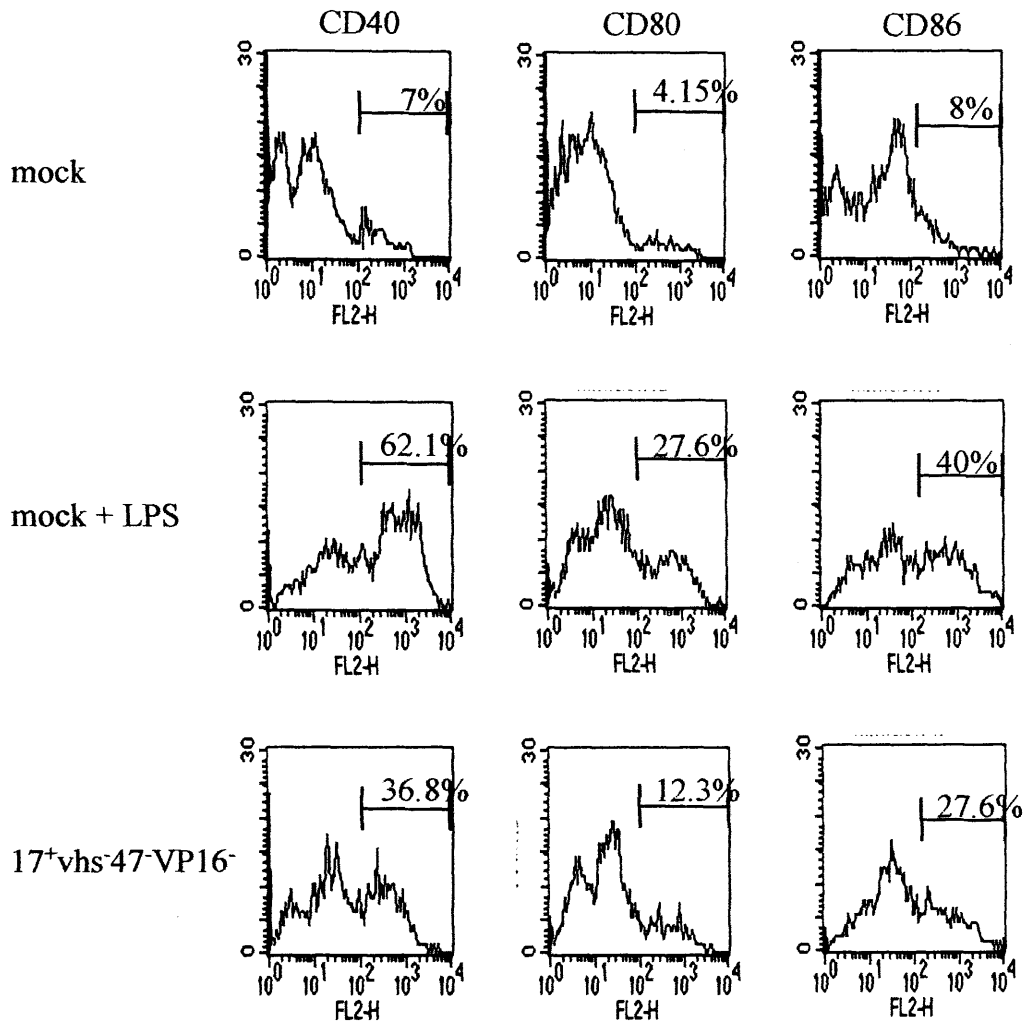


Figure 3.24: Effect of with 17⁺vhs-47-VP16⁻ infection on BMDC maturation

Level of expression of maturation markers on BMDC after infection at MOI1 with 17⁺vhs-47-VP16⁻ as compared to treatment with LPS.

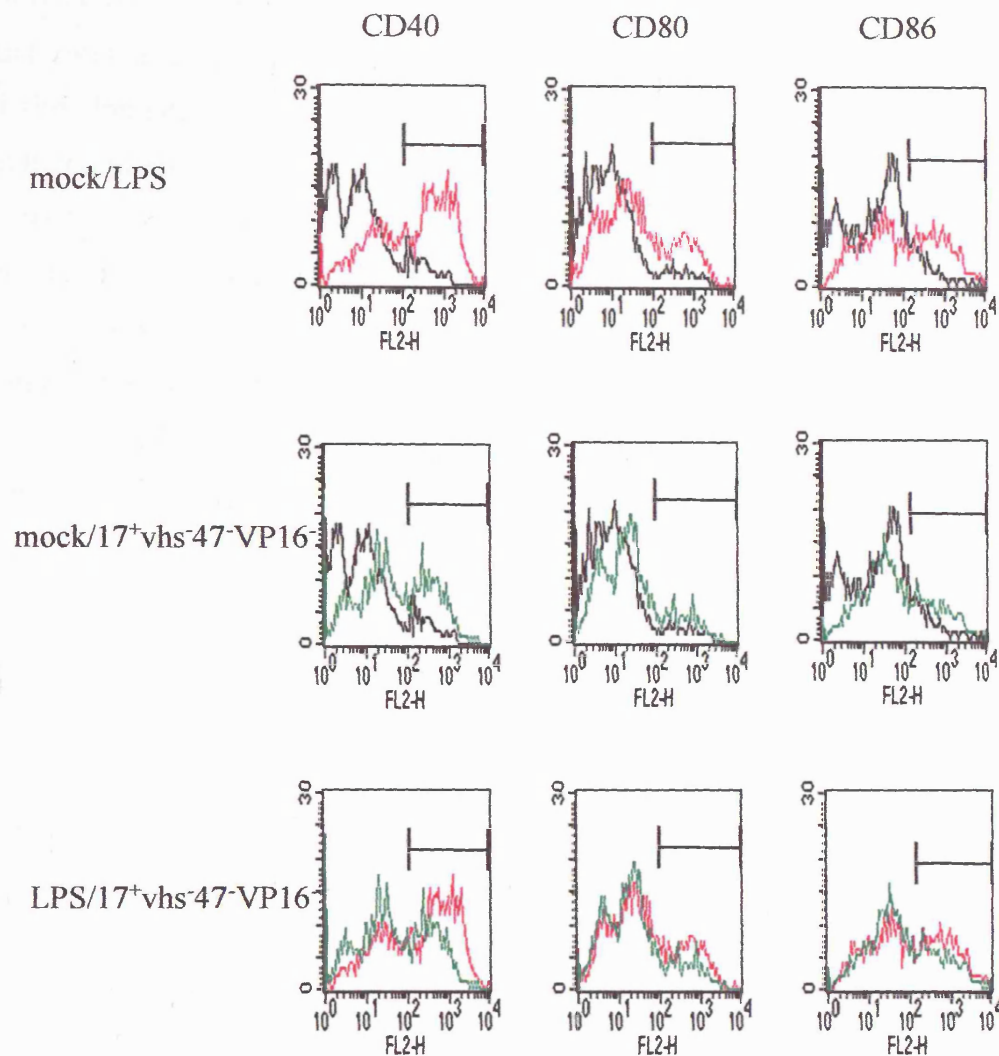


Figure 3.25: Effect of 17⁺vhs⁻47-VP16⁻ infection on BMDC maturation markers

Level of maturation markers expression on BMDC after 17⁺vhs⁻47-VP16⁻ infection (green), compared to the level of expression on immature uninfected cells (black) and on LPS mature uninfected cells (pink).

3.5.3 Effect of 17⁺vhs⁻47⁻VP16⁻ infection on DC maturation with LPS stimulation

As for the other backbones, the effect of the vhs- virus with the additional VP16 mutation was assessed on the ability of infected DC to mature upon LPS stimulation. Immature BMDCs were infected at MOI1 with 17⁺vhs⁻47⁻VP16⁻ and then cultured in presence of LPS, before measuring the level of CD40, CD80 and CD86 on the cell surface. The percentages of cells expressing high levels of maturation markers (Figure 3.26) were similar to those previously obtained upon virus only infection (~35% for CD40, 11% for CD80, 28% for CD86). Overlaying the profiles of mature cells with those of infected matured cells for each marker (Figure 3.27) demonstrated a very similar profile, indicating undisrupted DC function by this measure.

The 17⁺vhs⁻47⁻VP16⁻ virus gave good infectivity without interfering with DC maturation and therefore is a good candidate for use for delivery of antigens to DC for immunotherapeutic studies. In the in vivo situation it might be expected that injection of infected DCs or this virus should allow the cells to mature and travel back to the LN, unlike the previous viruses. This would be expected to trigger an immune response to both the virus and the products of the genes carried by it.

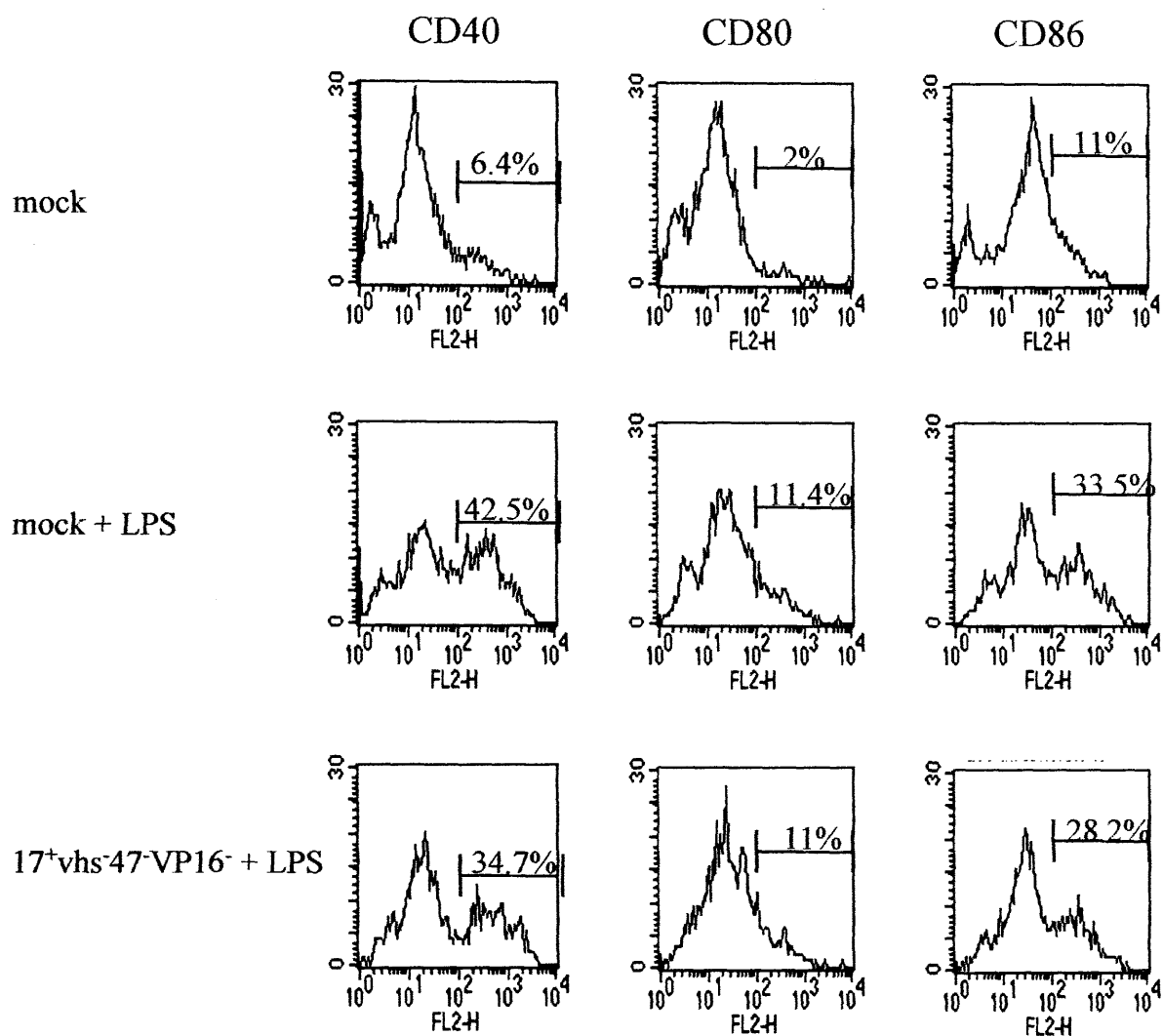


Figure 3.26: Level of maturation marker expression on BMDC infected with 17⁺vhs-47-VP16⁻ at MOI1 and stimulated with LPS.

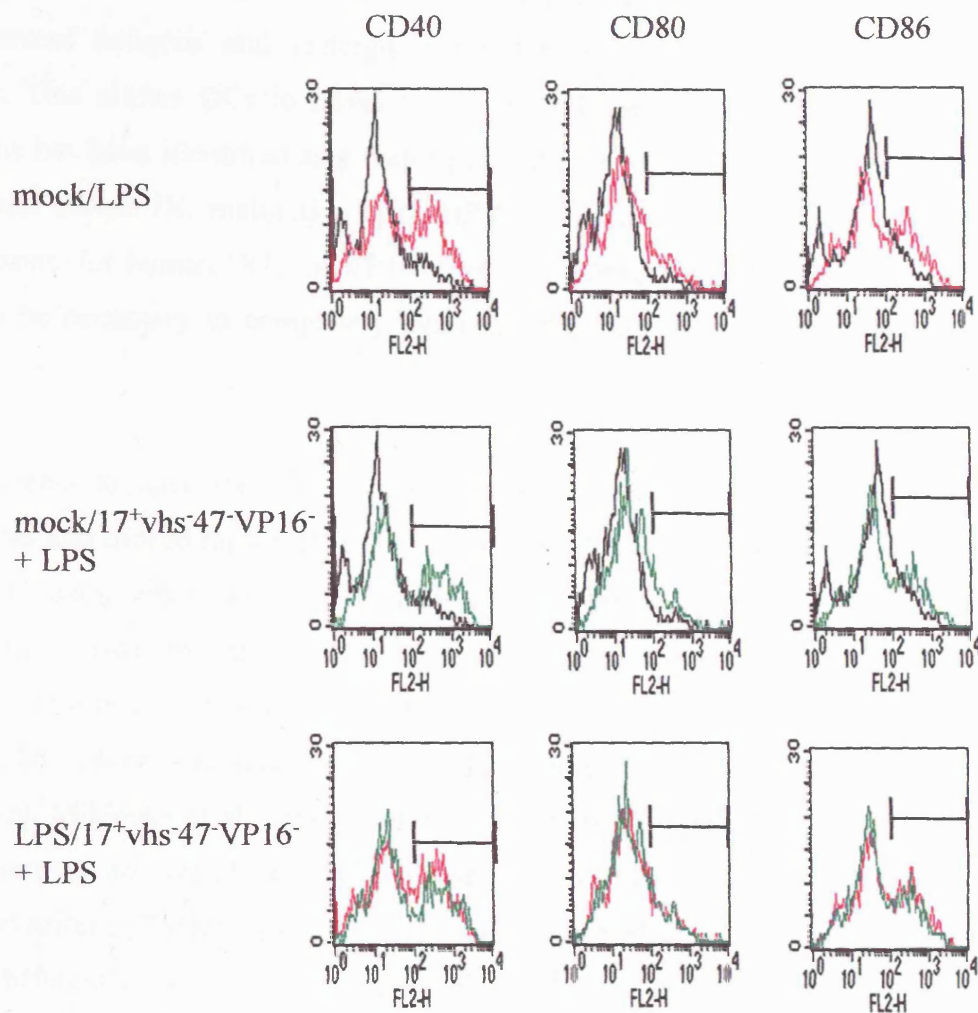


Figure 3.27: Effect of 17⁺vhs-47-VP16⁻ infection on maturation

Level of costimulatory molecules expression on the surface of BMDC infected with 17⁺vhs-47-VP16⁻ (green) and stimulated to mature, compared to the levels found on untreated BMDC (black), or uninfected LPS mature BMCD (pink)

3.6 Discussion

This chapter aimed at further investigating previous results using human DCs and, based on these, to define an optimal HSV-1 vector for gene delivery to mouse BMDC. For DCs to be used as vectors to stimulate an immune response, they must present antigens and undergo maturation with upregulation of surface markers. This allows DCs to travel to lymph nodes and interact with T cells. Here, vhs has been identified as a factor playing a major role in preventing both human and mouse DC maturation upon HSV-1 infection. The use of replication incompetent (for human DCs) or VP16- (for mouse BMDC) backbones has been found to be necessary to completely overcome the HSV-1 mediated maturation block.

HSV-1 seems to have naturally evolved to infect DC as an immune evasion mechanism and indeed highly efficient infection was obtained both in human and mouse DC, using either wild type or attenuated vectors. As previously reported, HSV-1 was shown to infect both human and mouse DCs non productively. Although there is a small amount of viral particles produced ~24hr post infection of human DC which is in agreement with other studies (Albers et al., 1989, Kruse et al., 2000, Mikloska et al., 2001, Pollara et al., 2003, Sarmiento & Kleinerman, 1990), there is an overall steady decrease in virus titre. The mechanism of immune evasion by infection of DCs is not unique to HSV1, but different viruses behave differently within these cells. Similarly to HSV1, vaccinia virus (Engelmayer et al., 1999), as well as influenza virus (Lopez et al., 2000) have been shown to abortively infect DCs, whereas Dengue virus (Ho et al., 2001, Libraty et al., 2001) and measles virus (Bhardwaj, 1997), can replicate within them. In addition, as an immune evasion mechanism, infection with measles virus (Grosjean et al., 1997) as well as vaccinia virus (Engelmayer et al., 1999) impairs the cells ability to mature, whereas infection with Dengue virus (Libraty et al., 2001) or influenza virus (Cella et al., 1999) do not prevent maturation.

It is still not clear what effect wild type HSV1 infection has on human DC. Controversial studies have reported both no effect on costimulatory molecules (Salio et al., 1999), cell maturation (Pollara et al., 2003), or even decreased

surface markers expression (Mikloska et al., 2001) upon infection, with differences occurring within an infected culture between the cells that do get infected and those that do not. Previous results from our laboratory (Samady et al., 2003) suggested that no effect is seen on infected human DC surface markers expression, and mouse DCs have now been shown to behave similarly. This supports the findings that contrarily to type I IFN producing DC ($CD11c^+CD11b^-B220^+$), myeloid BMDC ($CD11c^+CD11b^+B220^-$) do not respond to infection (Gilliet et al., 2002).

The response of the cell to virus infection and the ability of the cell to undergo maturation upon stimulation need to be considered separately. Although there is controversy over the first point, it is accepted (Pollara et al., 2003, Salio et al., 1999) that HSV infected DCs are prevented from undergoing maturation upon LPS stimulus, and infected mouse DC have now been shown to respond similarly. Interestingly, the impairment is not limited to the maturation process, but can also affect the T cell stimulatory capacity of already mature DC, (Kruse et al., 2000, Pollara et al., 2003, Salio et al., 1999).

The exact mechanism by which HSV1 prevents infected DCs from maturing upon LPS stimulation is still not understood, but it affects both cytokine release and surface molecule (MHC and maturation markers) upregulation. ICP47 and vhs are known to be involved in interfering with MHCI and MHCII presentation respectively. Considering that ICP47 does not interfere with mouse cell antigen presentation (Hill et al., 1995, Tomazin et al., 1996), the effect of removing this gene was only investigated from the infectivity point of view, and the slight improvement in maturation seen in BMDC upon infection with a 17^{+47} -vhs- virus can therefore probably be attributed to the removal of vhs.

Vhs has been identified as a key mediator in blocking DC maturation as removing vhs from wild type virus improved both human and mouse cells ability to respond to LPS stimulation and making the virus replication incompetent had no beneficial effect unless vhs was also deleted. This finding is not surprising considering the known functions of vhs and the role it plays in anti-HSV immune responses. Vhs has been categorised as an RNase or as a required subunit of an RNase complex

(Elgadi et al., 1999, Everly et al., 2002, Krikorian & Read, 1991, Sorenson et al., 1991, Zelus et al., 1996) and it specifically degrades mRNAs (Krikorian & Read, 1991, Kwong & Frenkel, 1987, Oroskar & Read, 1989, Zelus et al., 1996) probably by targeting translation initiation factors such as eIF4 (Feng et al., 2001). Vhs initially degrades both host cell mRNAs preventing peptide presentation and viral mRNAs favouring transition to expression of viral genes from later phases. Vhs activity is later blocked by binding to VP16 thus allowing undisturbed viral mRNA transcription and translation, and viral replication (Lam et al., 1996, Smibert et al., 1994, Weinheimer et al., 1992).

Vhs, in addition to ICP47, has been shown to interfere with MHCI presentation, but by inhibiting *de-novo* protein synthesis (Hill et al., 1994, Tigges et al., 1996). It also seems to be responsible for downregulation of MHCII chain presentation in glioblastoma cell by preventing MHCII chain formation (Trgovcich et al., 2002). Taken together these results agree with the findings that vhs may be responsible for preventing costimulatory molecules upregulation and suggest that this would be due to inhibition of maturation marker synthesis.

Because of the ability of vhs to interfere with peptide presentation, vhs⁻ viruses have mainly been studied as candidates for therapeutic or prophylactic vaccines against HSV1. Removing vhs from a replication incompetent (ICP8-) virus allowed stronger humoral and cellular responses against HSV1 corneal infection in a prophylactic vaccine (Geiss et al., 2000). Similarly, Keadle et al (Keadle et al., 2002) have found that removing vhs from either ICP8- viruses or wild type virus used as therapeutic vaccine, induced a stronger antibody response. Removing vhs therefore allows stimulation of a better immune response, in agreement with the fact that vhs- viruses interfere less with DC activity.

Vhs, therefore, plays a significant role in HSV-1 immune evasion, which seems to include prevention of maturation of infected DC. From our results it appears that vhs is not the only factor interfering with DC maturation, and the use of replication incompetent viruses seem to be necessary to completely overcome the maturation block in human cells, indicating that one of the IE genes may also takes part in the inhibition process. As replication incompetent vhs- HSV-1

vectors are non pathogenic, and can not reactivate from latency (Smith et al., 2000), these vectors appear good candidates for use in immunotherapy. Interestingly, in BMDC, the V422 mutation of the VP16 activation domain gave similar beneficial effects as for replication incompetent viruses in human DC. Less disabled viruses were not tested on human DC.

VP16, like vhs, is present in the tegument of the virus and is injected into the cell upon infection. VP16 is a viral transactivator, responsible for the formation of the initiation complex that triggers IE gene expression for lytic viral infection (Batterson & Roizman, 1983). Inactivation of VP16 is expected to attenuate the virus enough to render it safe and to reduce further replication, without having to delete additional immediate early genes. ICP0 can usually take over IE genes activation upon VP16 inactivation (Mossman & Smiley, 1999), but the vhs⁻VP16⁻ virus was still unable to replicate in BMDCs, accounting for a significantly attenuated virus.

BMDC infection with a vhs⁻VP16⁻ backbone was less toxic to the cells, which, together with the other beneficial effects of this virus, are not due to reduced infectivity. Expression of recombinant genes from the VP16 locus was found not to be very efficient, but inactivation of the transactivation domain did not seem to affect the virus ability to infect DCs or to express recombinant genes inserted in other positions. The fact that the vhs⁻VP16⁻ virus could induce maturation of the cell even without LPS stimulation, indicates that the virus evasion mechanisms can be overcome by removing vhs and mutating VP16 since the cell is now able to identify the presence of the virus and respond to it. Confirming this, no inhibitory effects were seen upon LPS stimulation.

The vhs⁻VP16⁻ backbone has therefore been shown to provide an attenuated vector, unable to replicate in BMDC, with reduced ability to express lytic genes, although capable of expressing recombinant genes, and allowing infected DC to undergo maturation upon stimulation. The aim of this chapter was to determine the appropriate backbone for use as a vector able to stimulate immune responses to delivered antigen encoding genes. Applying the data obtained from human DC to mouse BMDC indicated that the virus behaves similarly in both sets of cells,

and 17⁺vhs⁻47⁻VP16⁻ has been identified as a candidate vector for immunotherapy studies in mice. Upon *in-vivo* injection, the virus should efficiently target and infect local immature DC without disrupting their ability to undergo maturation, allowing them to travel back to the draining lymph nodes to initiate an immune response. Similarly, injection of infected DCs should be able to undergo the same process. This will be tested in later chapters.

Chapter 4

Ability of HSV-1 to migrate to the lymph node

4.1 Introduction

Dendritic cells are unique in their ability to stimulate naïve T cells. DC-T cell interaction is necessary to initiate an immune response and this specific function of DCs has made them key targets for vaccine development. T cell priming takes place in secondary lymphoid organs (lymph nodes and spleen) and because DCs are found scattered around the body in an immature form, their ability to migrate from their site of residence or from the site of infection to the lymph nodes or spleen is essential (Banchereau & Steinman, 1998). For migration to take place, the DC needs to be activated and to mature. Activation is a reversible state that is induced immediately following antigen uptake or stimulation and does not involve phenotypic changes in the cell. Maturation, on the other hand, follows activation and drives the cell into an irreversible state with upregulated costimulatory and migratory molecules.

In vivo, upon stimulus or infection, the immature DCs present take up the antigens or apoptotic cells containing the antigen by a process known as cross-presentation (Carbone et al., 1998) (Albert et al., 1998a, Albert et al., 1998b). After antigen uptake, the activated DCs lose their ability to respond to stimuli in favour of developing a costimulatory and migratory capacity. Maturation and migration of DCs are processes that take place concomitantly, allowing the cells to reach the LN in a fully mature state capable of both presenting the specific antigen and of activating the naïve T cells present. In addition to upregulation of costimulatory molecules (CD40, CD80, CD86), that allow the interaction with and activation of T cells, the maturation-migration process requires a shift of expression of CC chemokine receptors on the surface of the dendritic cell to allow migration to the LN (Sozzani et al., 2000). Immature DCs express CC receptors (such as CCR5) that bind inflammatory chemokines produced at the sites of infection (MIP1 α in the case of CCR 5). However, upon maturation the expression of these CC receptors is downregulated in favour of CC receptors that bind chemokines constitutively expressed in the lymphatics and LN. The most highly expressed of these is CCR7 that binds both the CC ligand CCL19 expressed by stromal cells and DCs in the lymph node paracortex, and CCL21 expressed by endothelial cells in the lymphatics and in high endothelial venules.

Release of these and other chemokines drive the DCs that express the appropriate receptors to move into the lymphatics and then into the LN (Dieu et al., 1998, Sozzani et al., 1998, Sozzani et al., 2000).

The LN is where an immune response is initially mounted. Lymph nodes are found at major intersections of the lymphatic system. They contain lymphocytes, macrophages and dendritic cells packed in organised sectors (figure 4.1). The LN is surrounded by a capsule from which trabeculae (connective tissue) penetrate and partition the node. There are three main layers that form the lymph node: the cortex, paracortex, and medulla, each containing different cell types. The cortex mainly contains B cells, macrophages and follicular dendritic cells arranged into follicles. These are either primary follicles, mainly containing B lymphocytes, or secondary follicles, consisting of aggregates of lymphocytes and containing a germinal centre (activated lymphocytes: plasma cells and memory B cells). T lymphocytes are mainly found in the paracortex, located beneath the cortex, together with interdigitating DCs that have migrated from peripheral tissue and some macrophages. Finally, the most central part of the LN, the medulla, contains lymphoid lineage cells, mainly antibody secreting plasma cells and macrophages.

The incoming antigen loaded mature DCs enter the LN through the capsule via the afferent lymphatic vessels that releases the lymph in the subcapsular sinus separating the capsule from the cortex. While passing through sinuses located in the cortex and paracortex, the lymph is filtered and depleted of any antigens, including antigen presenting cells, by a mesh of macrophages and dendritic cells present in these zones. Incoming DCs stop in the paracortex near high endothelial venules (HEVs) (Bajenoff et al., 2003, Mempel et al., 2004) where they become interdigitating DCs ready to scan the incoming Th cells and to interact with the Th cells present in the same area (Mempel et al., 2004). Once the cells have reached the lymph node, interaction and priming of T cells takes place in three phases (Mempel et al., 2004). Initially, short lived encounters of T cells and DC activate the T cells. The second phase sees stable interactions between the two cells and T cell cytokine release leading to the third phase of T cell proliferation and high cell mobility. The lymph depleted of antigen presenting cells further crosses the LN through the medulla, which contains mainly plasma cells, and finally leaves the

node enriched with lymphocytes, via the efferent lymphatic vessels in the hilus of the node.

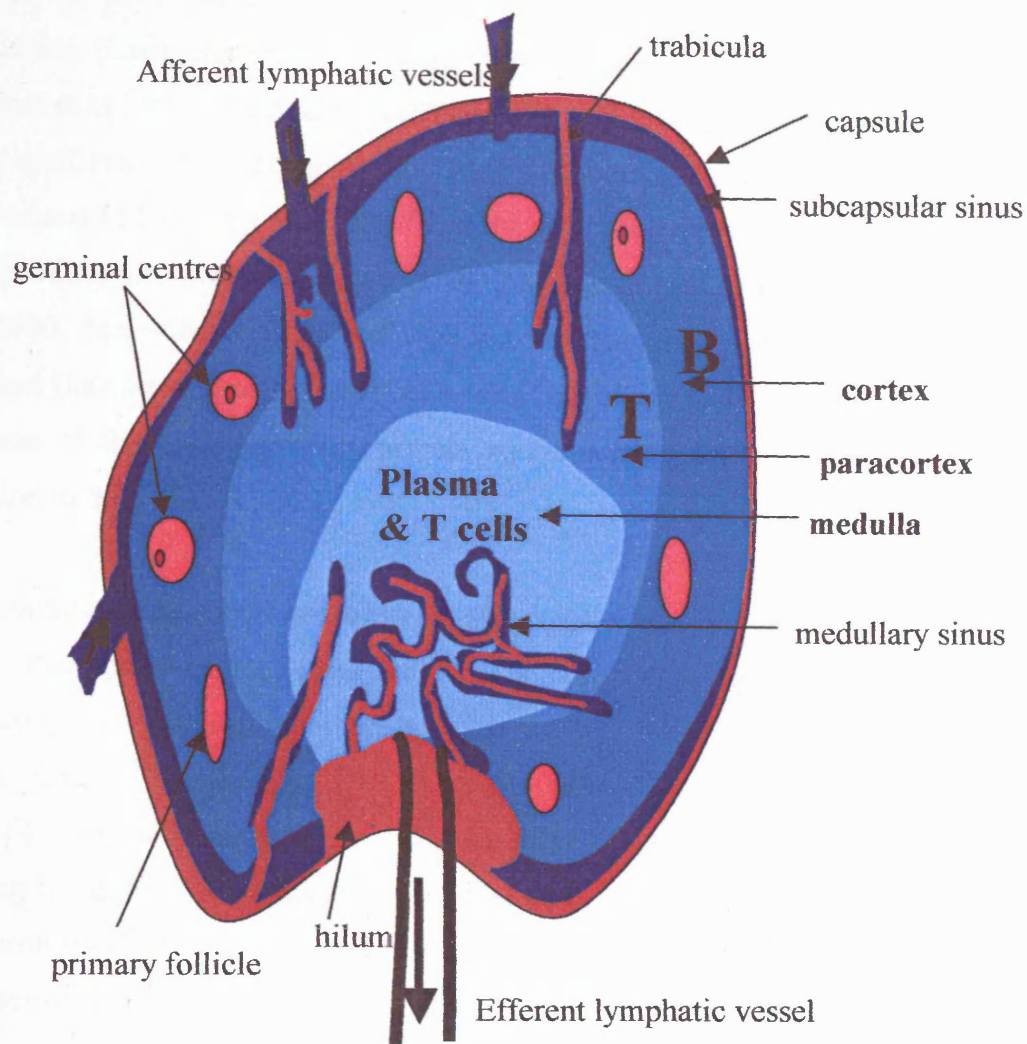


Figure 4.1: Structure of the lymph node

Diagram of a lymph node showing the main layers; the cortex, paracortex, medulla and the type of cells found within them. A connective tissue capsule surrounds the node. Afferent lymphatic venules pierce the capsule and release the lymph in the subcapsular sinus. The lymph then migrates through the node via the medullary sinuses and leaves via the efferent lymphatic vessel in the hilum. Trabeculae partition the node unevenly. The cortex contains mainly B cells arranged into primary follicles containing B cells, macrophages and follicular dendritic cells, or secondary follicles, containing a germinal centre. The paracortex area is mainly populated by T lymphocytes but also contains interdigitating dendritic cells. The medulla is composed of sparse lymphoid cells mainly plasma cells secreting antibodies.

The possibility of specifically targeting dermal DCs for vaccine purposes and their ability to migrate to the LN has already been shown using fluorescent dyes applied to the skin (Masurier et al., 1998, van Wilsem et al., 1994), gene gun immunisation (Larregina et al., 2001) or virus infection (Esslinger et al., 2003, Oberholzer et al., 2002). In addition, injection of DCs labelled ex-vivo with either dye (Bajenoff et al., 2003, Hermans et al., 2000, Labeur et al., 1999, Lappin et al., 1999), Indium-111 (Eggert et al., 1999) or by infection with a non-engineered or GFP expressing virus (Barratt-Boyces et al., 2000, Esslinger et al., 2003, Gardner et al., 2000, Masurier et al., 1998) has shown that after subcutaneous (s.c.), intradermal (i.d), as well as intramuscular (i.m.) injections cells accumulate in the T cell area of the draining LN, whereas intravenous injection (i.v) resulted in localisation to the spleen (Lappin et al., 1999).

An ideal viral vaccine should therefore be able to infect local DCs upon injection, allowing them to become activated and mature inducing upregulation of costimulatory and migratory molecules. The antigen loaded cells should then, therefore, travel to the draining LN to activate naïve T cells by presentation of both viral and recombinant antigens. HSV-1 antigens have already been identified in draining LN after footpad injection (Mueller et al., 2002), but these were found to have been transported following virus replication at site of infection. Although most routes of injection result in DCs presenting antigens in the LN, the strength of the immune response induced varies and the subcutaneous has been found to be among the routes achieving the strongest responses (Eggert et al., 1999).

Tracking of DCs infected with HSV-1 ex-vivo has not been possible so far because of the effect of the virus on cell maturation, but following HSV-1 subcutaneous footpad (f.p) injection, viral antigens have been found in the popliteal LN (Mueller et al., 2002). This has not always been correlated to the presence of virus (Jones et al., 2000, Mueller et al., 2002), instead, as expected, HSV-1 infection prevented maturation of DCs, and thus migration did not occur. The presence of antigen was therefore attributed to uptake by cross-presentation. Contrary to infection with HSV-1, infection of DCs with influenza or Sendai virus do not interfere with DC maturation (Hamilton-Easton & Eichelberger, 1995, Usherwood et al., 1999) and has been shown to allow infected DCs to migrate to

the LN. Therefore, having demonstrated that $17^+vhs^-47^-VP16^-$ infection allows DC maturation, migration of both ex-vivo and in-vivo infected DCs should be possible. This was tested in the following experiments.

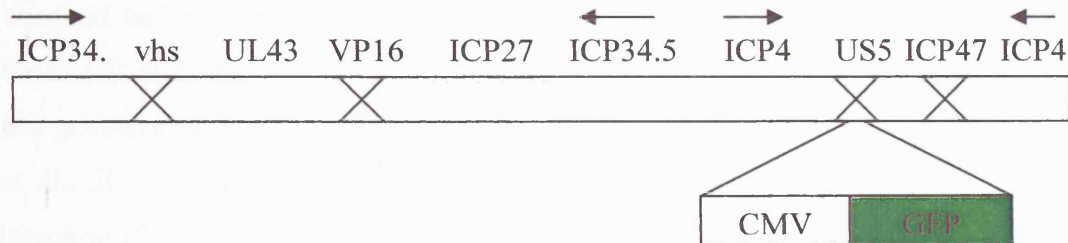
Virus used:

$17^+vhs^-47^-CMVGFP/US5 VP16^-$

vhs and ICP47 genes deleted

VP16 with V422 mutation

US5 inactivated by insertion of CMVGFP



X indicates a deleted or inactivated gene

→ indicates the direction in which the gene is expressed

4.2 Identification of DCs in the LN

4.2.1 Identification of ex-vivo infected DCs in the draining LN

The most practical way of following infected cells is to use a virus expressing a marker gene. 5×10^5 immature bone marrow DCs were infected for 4hr at MOI=1 with $17^+ \text{vhs}^- 47^+ \text{CMVGFP/US5 VP16}^-$, and then injected s.c. into mouse footpads. Taking into account previous studies (Barratt-Boyes et al., 2000, Nair et al., 2003) and anticipating that infected cells would be activated, and ready to respond to local stimuli, cells were not otherwise matured prior to injection. To ensure that cells were well infected and remained fluorescent for the duration of the experiment, a sample was kept in culture in vitro and GFP expression was checked before removal of the popliteal LN 24hr after injection. Many studies have looked at the timing of cells homing to the LN after injection, and although 6hr post-injection antigen presenting cells are already found in the LN (Bajenoff et al., 2003), maximal cell numbers have been detected between 24-48hr post-injection (Binder et al., 2000, Hermans et al., 2000, Lappin et al., 1999, Masurier et al., 1998, Oberholzer et al., 2002).

It was not possible to identify GFP fluorescent cells in whole LN or in LN sections, in both cases partly because of autofluorescence problems. There is a possibility that by the time the injected cells reach the LN, GFP has been processed by the cell and can therefore not be detected. To overcome this problem it was decided to track migration by loading the infected cells with a red fluorescent dye (CMTMR). However, unfortunately, still no fluorescent cells could be detected. Finally, LN sections were stained using an anti-HSV1 Ab. Considering that infected DCs were extensively washed before injection, no free virus should be present, and therefore any stain observed should represent injected cells that have migrated.

Mice were injected in the footpad with 5×10^5 immature DC or 5×10^5 DC infected at MOI1 with $17^+ \text{vhs}^- 47^+ \text{GFP/US5 VP16}^-$, and draining popliteal lymph nodes removed 24hr later. As it can be seen from figure 4.2, it was possible to detect HSV-1 antigen positive cells in what appeared to be the T cell area of the LN after injection of infected DC but not non-infected DCs. This implied that infected

immature DCs were able to induce and respond to maturation and migration stimuli upon injection. Direct comparison of infected and non infected DC injections should be sufficient to prove specificity of the stain. However, it appeared from additional controls (not shown) that when infected DCs were injected the DAB stain developed even when no antibody was added, albeit to a lesser extent.

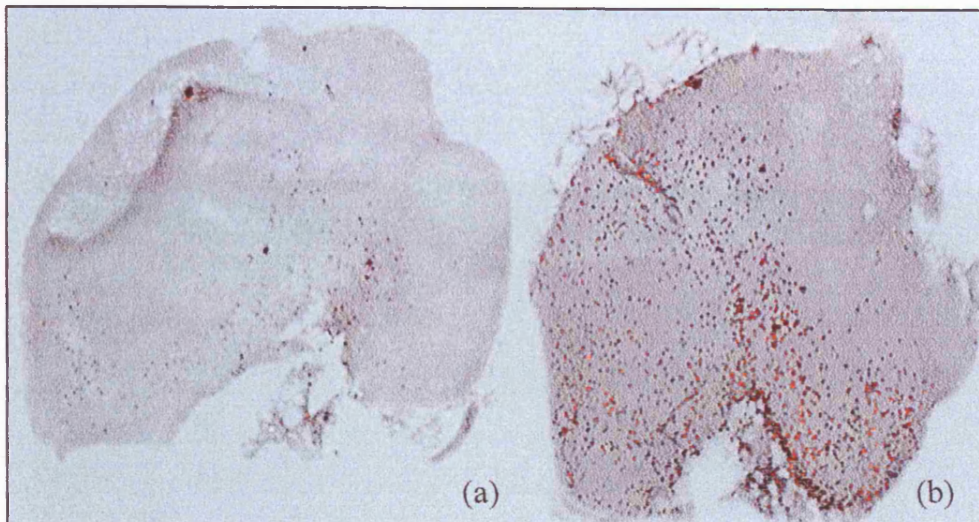
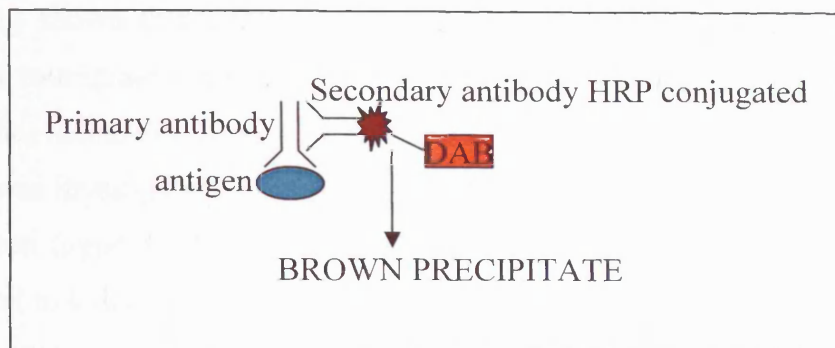


Figure 4.2: Detection of HSV-1 infected DCs in the LN

10 μ m cryosections of LN from mice injected in the footpad with 5×10^5 non-infected DC (a) or 5×10^5 DC infected MOI1 with 17^+ vhs $^-$ 47 $^-$ GFP/US5 VP16 $^-$ (b). LN were removed 24hr post-injection and stained with anti-HSV antibody detected with DAB staining.

This last observation indicated that the presence of virally infected cells caused a non-specific reaction with DAB. To further study this phenomenon, DCs and BHK cells were infected with 17^+ vhs $^-$ 47 $^-$ GFP/US5 VP16 $^-$ in vitro and DAB stain was then added to test for possible reaction of the virus with DAB (Figure 4.3). However, no reaction could be seen from either infected or non infected cells, suggesting that the staining seen in the LN is not due to the virus itself but possibly to some other factor found in the LN environment, maybe related to the activation of T cells.



Dendritic cells

BHK

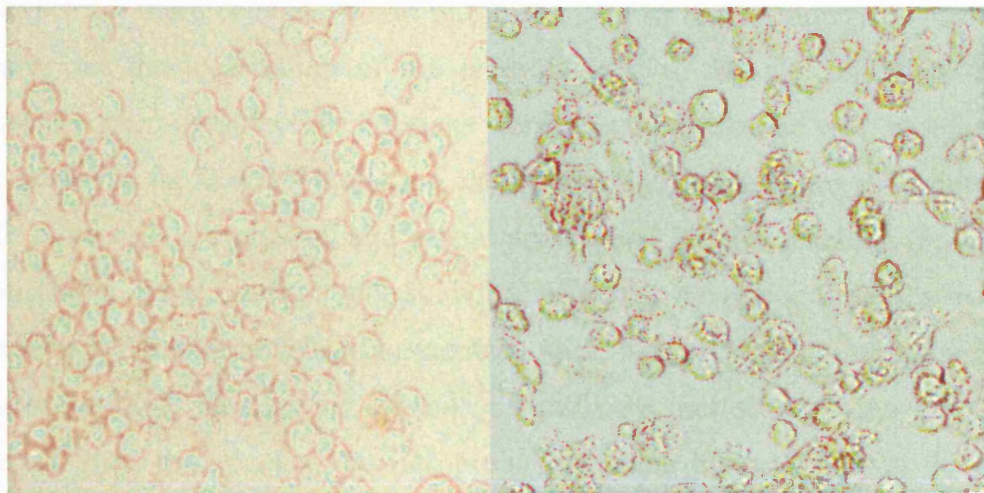


Figure 4.3: HSV-1 infected DCs and BHK cells with DAB stain added to them

Schematic representation of principle of DAB staining

20X photo of dendritic cells and BHK cells infected with 17⁺vhs⁻47⁻GFP/US5 VP16⁻. These cells had no primary or secondary antibody added to them, only the DAB stain was added to check for unspecific development of this stain due to presence of virus.

4.2.2 Identification of infected cells in draining LN after virus injection

Having shown that DCs infected with 17⁺vhs⁻47⁻VP16⁻ viruses maintain their ability to migrate to the LN, and having defined a potential (although not totally specific) method of identifying infected DCs in LN, migration of in-vivo infected DCs was investigated. Because following footpad injection of HSV-1 viral DNA has been found in LN as soon as 2hr post-infection (Mueller et al., 2002), it was decided to look at migration at both 6hr and 24hr post-injection. Mice were either left un-injected or were administered with 5×10^5 pfu of 17⁺vhs⁻47⁻GFP/US5 VP16⁻ in the footpad and draining lymph nodes removed after 6hr and 24hr. Figure 4.4 shows that HSV1 is only detected in sections from infected mice, and, as expected, more cells are found in the LN 24hr after infection. It is possible to clearly see the localisation of the infected cells to the paracortex of the LN especially 6hr post-infection. Sections were not counterstained with haematoxylin and eosin but the B cell follicles are clearly distinguishable in the 6hr sections. By 24hr more cells are present, and spread across the LN, also reaching the medulla where more naïve T cells can be found. Similarly to the previous results from DC injection, sections from LN of immunised mice, but not from unimmunised mice, reacted to DAB staining in absence of antibody (not shown). As before, the injected virus that reaches the LN, probably having infected DCs at the site of injection, seems to stimulate peroxidase activities in the LN. Comparing the two methods of injection it appears that neat virus inoculation results in a higher amount of virus present in the LN, although the two have not been quantified.

These results demonstrated the presence of viral antigens in the LN, which in the case of infected DC injection could only be due to trafficking of infected DCs. However, it had not been possible to specifically correlate the presence of virus with the arrival of infected DCs. To demonstrate actual trafficking of DCs, and to quantify any differences in the efficiency of the two immunisation methods, FACS analysis on LN cells was carried out to determine the numbers of DCs present.

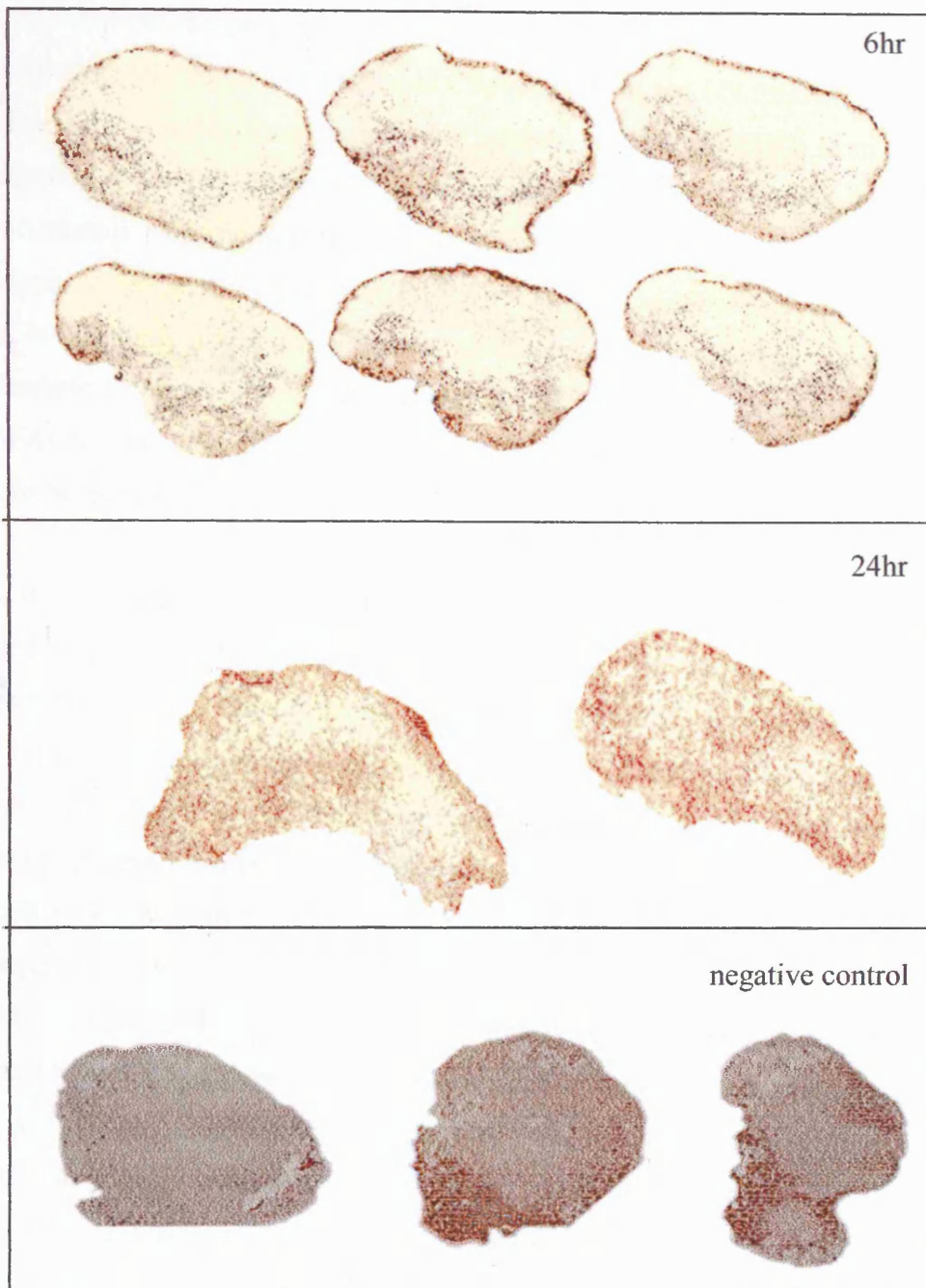


Figure 4.4: DAB staining of LN after footpad inoculation of mice with $17^+vhs^-47\text{-GFP/US5 VP16}^-$

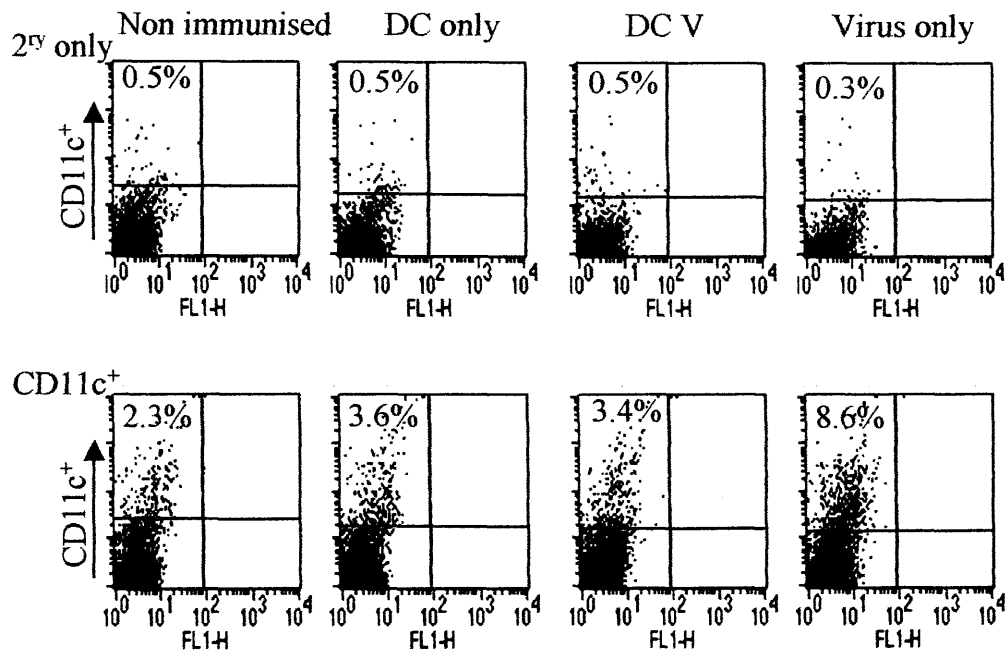
10 μ m cryosections of draining lymph nodes from mice injected in the footpad with 5×10^5 pfu $17^+vhs^-47\text{-GFP/US5 VP16}^-$ or non immunised (control). LN were removed 6hr or 24hr post-injection and sections were stained with anti-HSV antibody developed with DAB.

4.2.3 Detection of DC trafficking by determination of the cellular composition of the LN.

In these experiments the proportion of CD11c⁺ cells within the draining LN was assessed. An increase in the percentage of CD11c⁺ cells in the LN following DC or virus injection would indicate migration of infected and activated DCs from the site of infection to the LN. Other studies have looked at differences in the LN cell population, mainly identifying incoming fluorescently labelled cells (Labeur et al., 1999, Lappin et al., 1999, Masurier et al., 1998, van Wilsem et al., 1994). However, in this study it was decided not to use fluorescent dyes because it would not be possible to track cells infected directly in vivo. In addition, considering previous FACS data, GFP expression from the 17⁺vhs⁻47⁻VP16⁻ backbone may be too weak to be detected.

Groups of 9 mice each were injected s.c. in one footpad with either DC (DC only), DC infected with 17⁺vhs⁻47⁻GFP/US5 VP16⁻ at MOI 1 (DCV), neat virus (V only) or non immunised (n.i.). The draining popliteal LN from the nine mice were removed 24hr post injection and pooled together, crushed and stained for anti-CD11c for FACS analysis. As shown in figure 4.5a, it appears from all three experiments performed that resident CD11c⁺ cells usually account for ~2% of the total LN cell population. In two out of three experiments, a ~50% increase in the CD11c⁺ population was noticed 24hr after injection of immature DCs (becoming ~3% CD11c⁺ cells), and surprisingly, since viral infection is expected to be activate the DCs, this increase was not higher after injection of infected DCs. Interestingly, however, upon immunisation with neat virus the percentage of CD11c⁺ cells in the LN increased remarkably compared to the non immunised, DC, and DCV mice, reaching 7% of the total population.

Experiment 1



Experiment 2

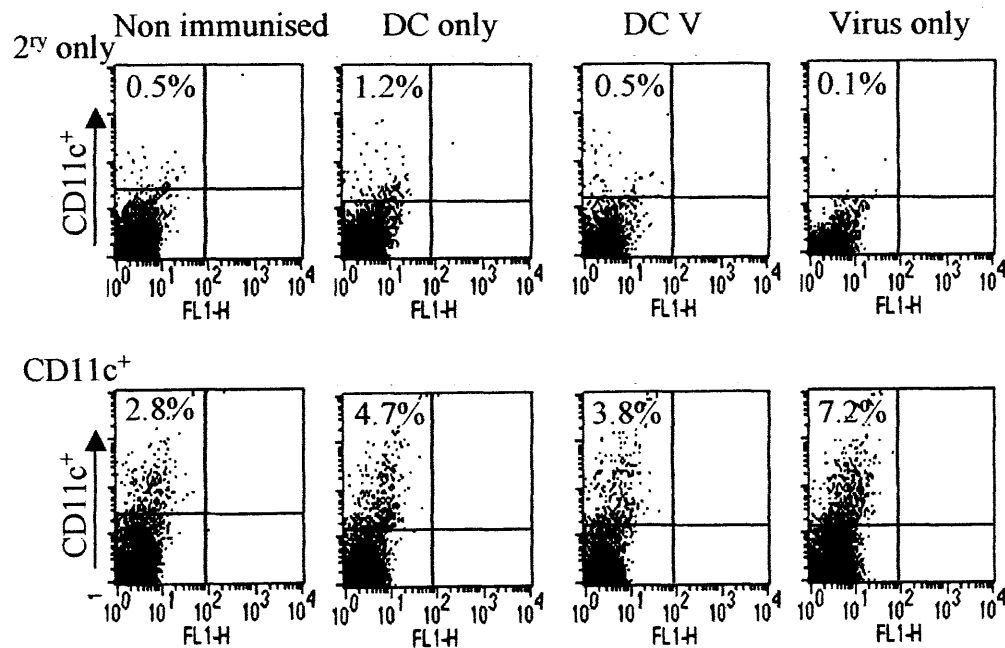


Figure 4.5(a): Detection of CD11c⁺ cells in LN following immunisation of mice

Experiment 3

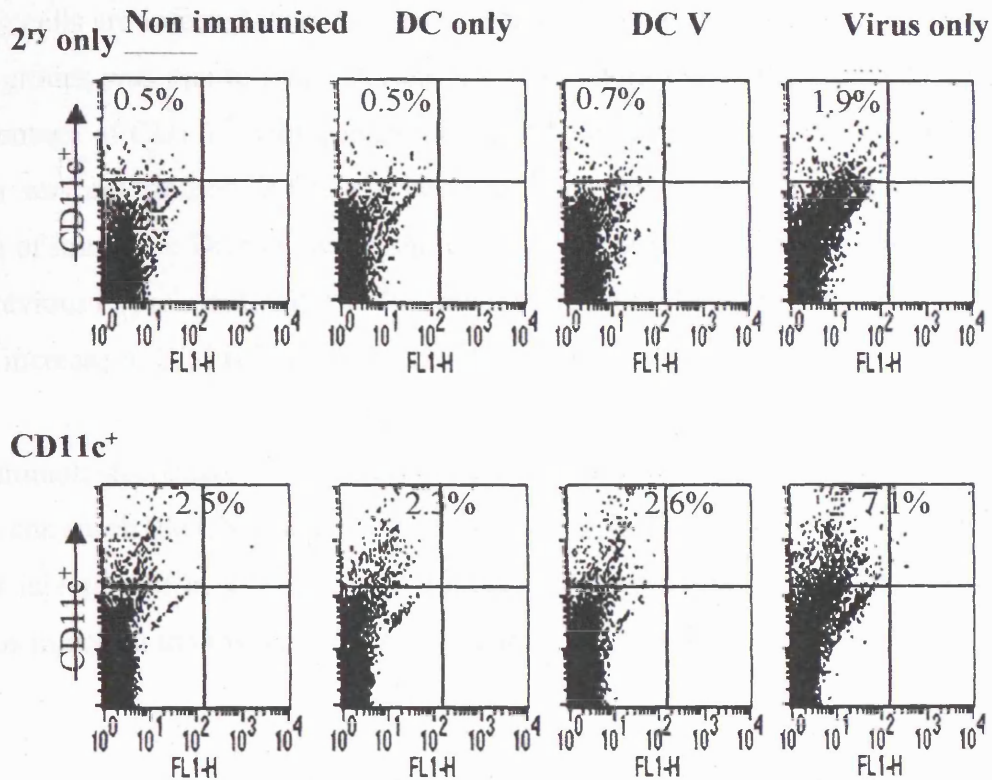


Figure 4.5 (a) continued

FACS dot plots from three replicate independent experiments showing the percentage of CD11c⁺ cells found in the total population of draining LN of mice injected with nothing (non immunised), 5×10^5 immature DC (DC only), 5×10^5 immature DCs infected with 17⁺vhs-47-GFP/US5 VP16⁻ (DCV), or with 5×10^5 pfu of 17⁺vhs-47-GFP/US5 VP16⁻ (virus only). The percentages of cells stained with the anti-CD11c⁺ antibody are compared to the percentages of positive cells obtained by addition of secondary antibody only.

These differences in cell population are shown more clearly in figure 4.5b, where the average of the three independent experiments is presented. As suggested from the staining of LN sections, injection of neat virus resulted in higher accumulation of DCs than injection of infected cells, although this method did not show that the incoming cells are infected. In order to understand whether the difference between the two groups was due to inappropriate in-vivo maturation of the injected cells, the percentage of CD11c⁺ cells detected in the LN after immature and mature DC injection was determined. As it can be seen from the FACS data (figure 4.6), injection of immature DCs increased the CD11c⁺ LN population by ~50-100%, as in the previous experiment, but if DCs were matured with LPS prior to injection, a ~ 200% increase in CD11c⁺ cells was found in the LN.

The immunohistochemistry and FACS results taken together show that viral antigens can reach the LN and there is an increase in the CD11c⁺ population in the LN after injection of in vivo or ex-vivo infected DCs and that this is higher after neat virus injection than when injecting infected or mature DCs.

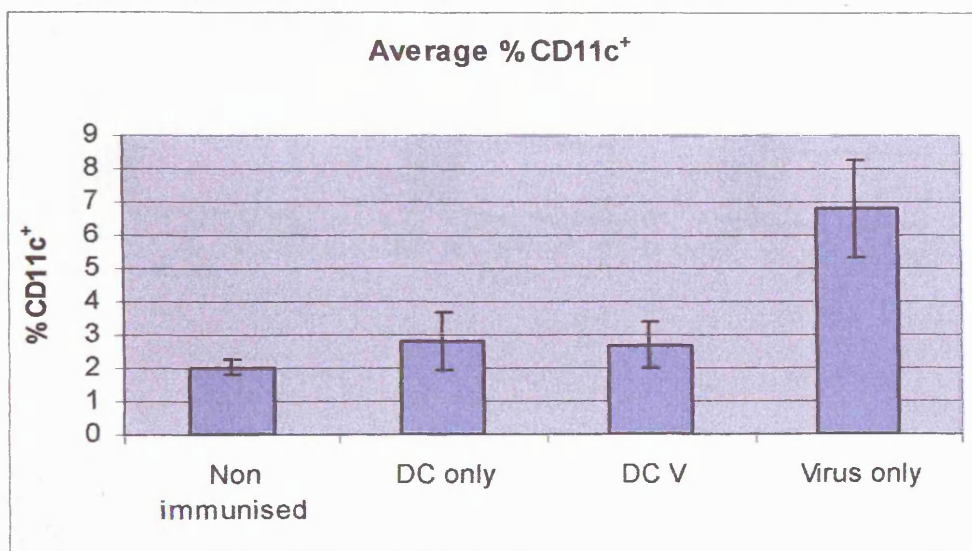
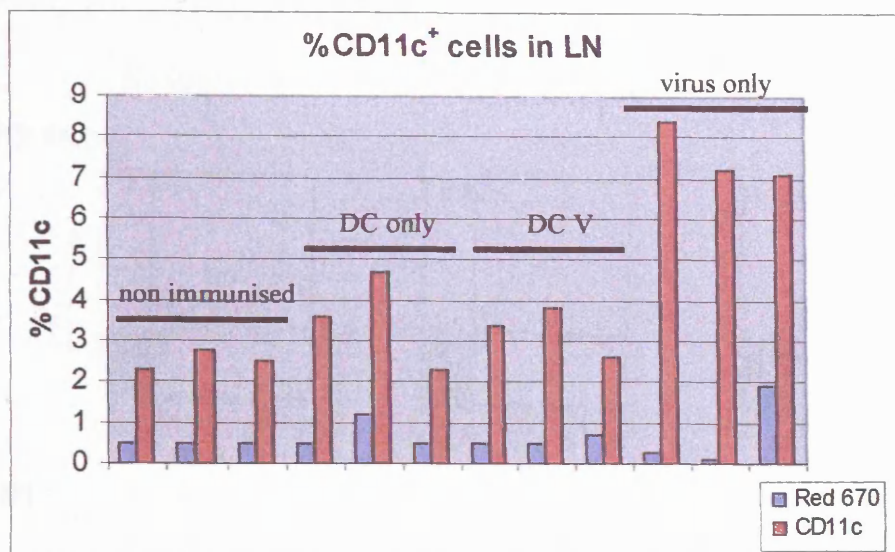


Figure 4.5 (b): Detection of DC in LN after inoculation of mice

Graphic representation of data shown in figure 4.5 (a) percentage of CD11c⁺ cells present in the draining LN of mice. Values obtained by addition of secondary antibody only (Red670) are compared to the percentages obtained by staining with anti-CD11c⁺ antibody. Values for the three independent experiments are shown. (b) Average of CD11c⁺ values obtained from the three independent experiments. Percentages obtained from staining with secondary antibody only were subtracted to the percentages obtained after CD11c⁺ stain. Error bars represent the standard deviations.

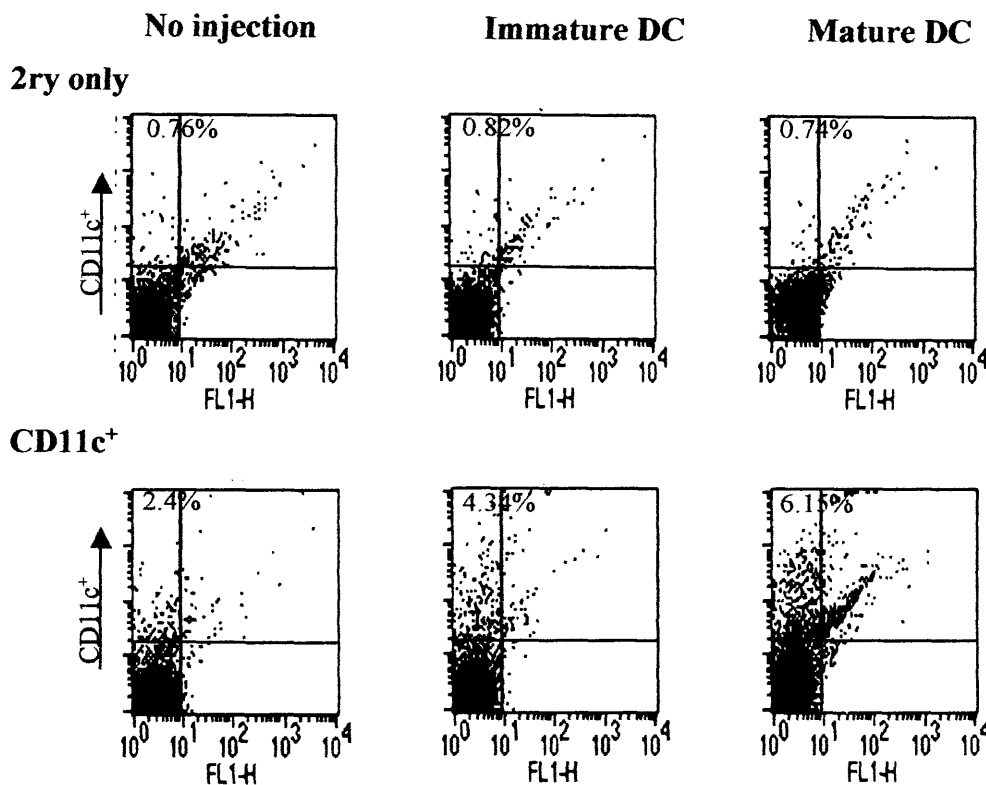


Figure 4.6: Detection of DCs in LN with or without activation of DCs prior to footpad inoculation

Comparison of the percentage of CD11c⁺ cells present in the draining LN in non injected mice, and after injection of 5×10^5 immature or mature DC in the footpad. Lymph nodes pooled from 9 mice either non-immunised, or immunised with immature DC or LPS matured DC were removed 24hr post-injection and the percentage of total CD11c⁺ cells present in the node (CD11c⁺) was determined and compared (as negative control) to the percentage of cells resulting positive by addition of only secondary antibody (secondary only).

4.3 Discussion

In vivo, dendritic cells are found in the immature state and are stimulated to undergo maturation upon antigen uptake. This modifies the pattern of cell surface receptors by promoting expression of molecules that allow cell migration to the draining LN and interaction with T cells. The 17⁺vhs⁻47⁻VP16⁻ backbone has been shown to allow infected DCs to undergo maturation when stimulated to do so, it was therefore important to determine whether infected cells also retained their ability to migrate to LN and initiate a response against recombinant antigens carried by the virus. Injection of infected DCs and neat virus resulted in viral antigen being found in the LN, this being higher after virus injection, and similarly injection of infected cells induced lower CD11c⁺ draining in the LN than neat virus.

Injected DCs have previously easily been tracked using fluorescent dyes (Bajenoff et al., 2003, Barratt-Boyes et al., 1997, Hermans et al., 2000, Lappin et al., 1999, Masurier et al., 1998), GFP expressing viruses (Esslinger et al., 2003, Gardner et al., 2000), or by labelling with Indium-111 (Eggert et al., 1999), all resulting in identification of the injected cells in the T cell area of the draining LN. Direct tracking of injected virus using GFP expressing HSV1 infected DCs has not been possible in this study due to the low level of GFP expressed from the particular backbone used. Although it is clear that activated T cells appear in the draining LN shortly after infection (Coles et al., 2002, Mueller et al., 2002), whether the antigens reach the LN from infected cells or by cross presentation at the periphery has been controversial. Early reports suggested that following i.d. injection, some infectious virus could be recovered in the draining LN and this was accompanied by an increase in the LN DC population (Sprecher & Becker, 1989), implying that the virally infected cells do undergo migration. Similarly, a study has recently shown that viral DNA can be detected in draining LN as soon as 2hr after s.c. footpad injection of HSV1, but this decreased with time, while antigen presentation increased, indicating that both infected DCs and DCs that have taken up viral antigens from infected cells migrate to the LN (Mueller et al., 2002). On the other hand, Jones et al (Jones et al., 2000) could not detect infectious viral particles or viral DNA in the LN at various times following f.p. infection, or flank

scarification (Mueller et al., 2002). Even in these last two reports, a CTL response could always be seen, suggesting cross presentation at the site of infection. In this chapter, migration of infected DCs to the LN was detected by staining for HSV 1, which can only detect the presence of viral antigens but not track infected DC. However, the detection of staining after injection of infected cells, where no free virus should be present, suggests that infected DCs can migrate to LN.

The results presented here are in line with those of Jones et al (Jones et al., 2000) as, unlike in other studies, DCs have been infected with a virus that does not prevent maturation, thus allowing cell migration. It would have been appropriate to include an additional control using wild type virus to prove that travelling of infected cells is possible only when the appropriate deletions to allow DC maturation are present in the backbone. With this control it would have been possible to conclude that migration of HSV-1 infected DCs is possible only if maturation of the cell is not prevented, in which case cross presentation takes place at the site of infection. This can be further seen using viruses that do not disrupt the ability of DCs to mature, such as influenza and Sendai virus, which have both been shown to infect DC inducing their migration (Hamilton-Easton & Eichelberger, 1995, Usherwood et al., 1999).

The fact that sections reacted to DAB (even in absence of primary antibody), only in the presence of virus following either infected cells or neat virus injection, is an indication of the arrival of viral antigen in the LN. Having proved that the virus itself does not react with DAB, a reaction must be stimulated by the virus in the LN. The in-vivo or ex-vivo infected DCs that reach the LN stimulate T cells resulting in IFN γ release. IFN γ has been shown to be sufficient to activate primary macrophages in a Myd88 dependent way (Shi et al., 2003), resulting in macrophage gene expression and NF-kB activation with production of reactive oxygen intermediates (ROIs) (Shi et al., 2003). These could develop DAB staining. Alternatively, considering that infected cells start reaching the LN within 6hr of injection (Bajenoff et al., 2003, Mueller et al., 2002, Olasz et al., 2002), there could be some minimal viral replication taking place in the LN in the mature DCs resulting in macrophages being activated and producing ROIs from mitochondrial oxydative stress (Mogensen et al., 2003). Although non productive

infection of DCs by 17⁺vhs⁻47⁻VP16⁻ has already been shown, macrophage activation only requires HSV1 IE and/or E genes (Mogensen et al., 2003), which can still be produced by infected mature DCs (Kruse et al., 2000) in the LN, although full viral replication and packaging of infectious particles does not take place.

Despite the unexpected DAB staining, it is possible to conclude from these results that DCs infected with the 17⁺vhs⁻47⁻VP16⁻ backbone are able to migrate to the LN, as well as the suggestion of the initiation of an immune response by the activation of T cells.

The identification of migrated cells within a LN focuses on a very restricted number of cells. In fact, DCs as shown here and by others (Barratt-Boyes et al., 2000, Binder et al., 2000) account for ~2% or less of a LN population. A general increase in LN cellularity (Binder et al., 2000, Mori et al., 2001, Mueller et al., 2002) is an indication of the stimulation of an immune response. Following infection, both migrating antigen presenting DCs (Binder et al., 2000, Gardner et al., 2000, Lappin et al., 1999, Mori et al., 2001) and naïve T cells (Mempel et al., 2004, Mori et al., 2001) drain to the local LN. The number of antigen presenting cells that reach the LN is a very important factor in the determination of the type of response induced: a large number of cells result in a sustained stimulation through single or successive DC-T cell interactions, whereas low numbers of poorly stimulatory DCs will induce abortive T cell proliferation and tolerance (Lanzavecchia & Sallusto, 2001). Following s.c. injection of DCs, the number of injected cells that are found in the draining LN has always been quite low, accounting for 0.1%-10% of the initially injected cells (Barratt-Boyes et al., 2000, Hermans et al., 2000, Labeur et al., 1999, Lappin et al., 1999, Olasz et al., 2002). Such low efficiency of injected DC migration partly depends on the route of administration. S.c. injection results in a high proportion of cells remaining at the site of injection (Eggert et al., 1999, Labeur et al., 1999, Lappin et al., 1999, Olasz et al., 2002), but, despite this, when compared to other routes, s.c. inoculation has been identified (Bedrosian et al., 2003, Lambert et al., 2001, Onaitis et al., 2002) to be able to induce one of the strongest CTL responses (Eggert et al., 1999, Lappin et al., 1999, Mullins et al., 2003), with only intranodular injection giving a

better response. The increase seen here in the CD11c⁺ population are higher than previously reported, considering that no DC enrichment step was used, and that the increase is noticeable over the whole CD11c⁺ population. Sufficient DCs would therefore be expected to be present in the LN to stimulate CTL responses rather than inducing tolerance.

To further confirm that the increase in the CD11c⁺ LN population was due to draining of skin derived DCs, it would have been appropriate to identify which of the five different types of CD11c⁺ cells present in the LN (Henri et al., 2001) accounted for the increase by staining for additional markers such as CD11b, B220, and CD8 α .

Both staining of LN sections and FACS data showed that neat virus injection resulted in higher accumulation of DCs in the draining LN than infected DC injection. The most important factor in DC trafficking is the maturation state of the cells. Ex-vivo maturation of the DCs prior to injection should not be necessary (Barratt-Boyes et al., 2000, Nair et al., 2003), and results from the previous chapter had shown that after infection with 17⁺vhs⁻47⁻VP16⁻ most of the cells were still immature. It was assumed that ex-vivo infected cells would be in an activated state, having taken up a foreign antigen, and that upon injection they would undergo self-maturation (Barratt-Boyes et al., 2000, Nair et al., 2003) such that they would react as well as in-vivo infected cells to local stimuli, resulting in similar trafficking to the LN. Although a certain degree of maturation did take place upon injection, accounting for an increase in the LN CD11c⁺ population, similarly to that found in other studies (Labeur et al., 1999, Lambert et al., 2001, Mackey et al., 1998), this migration was not as strong as seen after injection of mature cells.

The level of maturation of the ex-vivo infected cells therefore seems to explain the reduced flow of CD11c⁺ cells to the LN compared to neat virus injection. Migration of mature infected cells was not investigated, but considering that infection did not confer any increase in the trafficking of immature DC, both infected and uninfected DCs might benefit from a maturation step prior to

injection. Ex-vivo manipulated cells therefore retain their ability to migrate efficiently, but injection induced maturation does not result in the cells maturing completely, resulting in reduced trafficking to the LN compared to in-vivo infected cells. Comparison of ex-vivo and in-vivo infection methods has previously reached the same conclusion (Esslinger et al., 2003), thus indicating that ex-vivo infected cells do not respond as well as in-vivo infected cells to local stimuli encountered upon injection.

The previous points demonstrate that the methods used for the injection of DCs gives considerable variability in the results, making this route of administration not as efficient or reproducible as neat virus injection. Factors such as the efficiency of infection, the extent to which manipulation affects the maturation status of the injected cells, as well as DC in vitro culturing conditions and purification methods, may all be important variables that influence the efficiency of trafficking and reproducibility of the assay.

The data in this chapter suggests that the 17⁺vhs⁻47⁻VP16⁻ viral backbone can infect DCs without disrupting their ability to migrate to the LN and thus initiate an immune response. In addition it can be speculated, by comparing the efficiencies of migration, that neat virus injection might result in a stronger immune response than through the use of ex-vivo infected DC.

Chapter 5

Ability of HSV-1 to induce CD8⁺ CTL immune responses

5.1 Introduction

Immune responses are stimulated in the lymph nodes (LN) by arrival of antigen loaded dendritic cells (DC). Here, the mature DCs form clusters (Bajenoff et al., 2003, Ingulli et al., 1997) and prime naïve T cells that soon migrate to the spleen, where their proliferation takes place (Coles et al., 2002, Mueller et al., 2002). The previous chapter has shown an increase in LN DC cellularity following injection of ex-vivo infected DCs, or virus, suggesting that both techniques may lead to the induction of an immune response. The stimulation of CTL responses against a recombinant antigen has been shown to be possible following arrival in the LN of either peptide pulsed DCs (Eggert et al., 1999, Hermans et al., 2000), plasmid loaded DC (Liszewicz et al., 2001), or DCs infected in vivo with replicons (Gardner et al., 2000). Similarly, ex-vivo virally infected DCs as well as those targeted in vivo by recombinant viruses such as lentivirus (Esslinger et al., 2003) or adenovirus (Nakamura et al., 2002) have been shown, following arrival in the LN, to stimulate antigen specific responses. When using viral vectors, in addition to the response against the recombinant protein, anti-viral responses are usually also stimulated. In particular, footpad injection of (Coles et al., 2002) or scarification (Mueller et al., 2002) with HSV1 have been shown to stimulate anti-HSV responses in the LN initiated by CD8 α cells (Smith et al., 2003). In the construction of a viral vector, both types of immune responses need to be considered to ensure a balance and to avoid the anti-viral response overriding the specific targeted one.

Each pathogen has an immunodominant antigen, as well as immunodominant peptides within it (different for CD4, CD8, or B cells), towards which immune responses are mainly targeted. Vaccine therapies using DCs have focussed on the specific presentation of these immunodominant peptides for stimulation of specific responses. However, the use of the full length antigen to load DCs may be advantageous. Identification of CTL epitopes is very laborious though necessary, for each HLA allele if peptides are to be used. Expressing full-length genes in DCs allows the HLA profile of the patient to be ignored. Moreover, some individuals are tolerant to the immunodominant epitope, therefore subdominant epitopes should preferably also be identified (Cibotti et al., 1992). However, using

the full-length gene, multiple epitopes (including secondary ones) are naturally presented. By this means MHCII epitopes are presented in addition to MHCI, stimulating both CD8⁺ CTL cells and CD4⁺ Th cells (Hamel et al., 2002, Wan et al., 1999), which may therefore result in an increased response. Taking into account the data presented in previous chapters, the 17⁺vhs⁻47⁻VP16⁻ backbone appears to be the most appropriate vector for DC based therapy.

Replication deficient HSV1 viruses have already been studied for the induction of CTL responses to recombinant antigens (Brockman & Knipe, 2002), or to bacterial infection (*L.monocytogenes* expressing OVA) (Lauterbach et al., 2004), and have even been shown to confer partial protection to Simian immunodeficiency virus when encoding both Env and Nef genes (Murphy et al., 2000). Additionally, HSV-1 has been used as an amplicon vector where injection of ex-vivo infected DCs has been shown to be efficient in inducing an immune response to prostate cancer (Willis et al., 2001). Alternatively, in vivo injection of an HSV1 amplicon carrying the HIV gp120 gene has also resulted in successful stimulation of a specific CTL response (Hocknell et al., 2002). Together, this data indicates the potential for HSV-1 vectors to stimulate an immune response either by direct injection or by injection of infected DCs.

In this chapter, influenza nucleoprotein (NP) is used as a test gene to evaluate the ability of the 17⁺vhs⁻47⁻VP16⁻ backbone to induce an immune response in mice following subcutaneous footpad injection of ex-vivo or in vivo targeted DCs. As previously shown in this work, as well as by others (Larsson et al., 2000), injection of mature cells results in a greater degree of migration to the LN but, to avoid excessive manipulation of the cells, as well as possible non specific responses caused by LPS, the cells used for ex-vivo infection were not subject to maturation. However, as a positive control, the cells pulsed with the NP peptide were previously matured with LPS to ensure maximal presentation. This chapter will therefore focus on the possibility of inducing an anti-NP CTL response in comparison to the responses after immunisation with either naïve mature DCs, mature peptide pulsed DCs, DCs infected with 17⁺47⁻NP/vhsVP16⁻ at MOI=1, or by injection of 17⁺47⁻NP/vhsVP16⁻ directly.

Different cytokines may be used in conjunction with a vector to increase (eg. GMCSF) or inhibit (eg. IL-10) immune responses. When compared, to other cytokines, GMCSF has generally been demonstrated to be the strongest enhancer (Weiss et al., 1998). GMCSF is a 23kDa, 127aa glycoprotein produced by many cells in the body that enhances the maturation of DC cell precursors. GMCSF promotes upregulation of co-stimulatory molecules and MHC presentation on APC (Morrissey et al., 1987, Wang et al., 2000) and also stimulates cell migration to the LN by enhancing expression of CC chemokine receptor 7 on DCs (Nakamura et al., 2002). An increased cellularity both in the spleen and in the LN seen after continuous or discontinuous injection of GMCSF has been shown to be due to recruitment of cells of the myeloid lineage, produced by the bone marrow (Basak et al., 2002, Miller et al., 2002), but with different stimulatory properties to the usually matured myeloid cells (Miller et al., 2002). For example these cells release less IFN γ but much higher levels of IL6.

Many studies have focussed on the advantages of using GMCSF to recruit DCs for tumour therapies. In particular, the use of HSV1 vectors expressing cytokines (IL2 or GMCSF) has been exploited to enhance the killing of tumour cells by virus replication (Liu et al., 2003, Loudon et al., 2003, Parkinson et al., 2003). The ability of this cytokine to enhance a response to various infections such as chlamydia (Lu et al., 2002), or plasmodium yoelii (Weiss et al., 1998) has also been demonstrated.

Incorporation of GMCSF into a vaccine vector therefore aims to enhance the activation of antigen presenting cells and antigen presentation to T cells. Many studies have delivered adjuvant cytokines as a plasmid or recombinant protein. However, expression from a virus has several advantages including a longer time of presence and lower cost. In addition, co-localisation of the antigen and the enhancer is important, as proved by experiments with BCG vaccine (Lu et al., 2002, Wang et al., 2002), and since HSV1 can easily co-express multiple proteins, this chapter will assess the effect of co-expressing GMCSF and NP on the CTL response induced.

Viruses used in this chapter

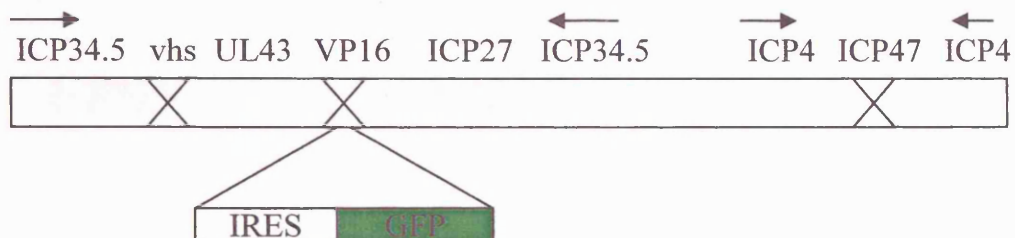
This diagram indicates the progression of virus construction
Specific deletions and inactivations of each backbone are specified

X indicates inactivity of the gene either by deletion or inactivation
→ indicates the direction of gene expression

17⁺vhs⁻IRESGFP/VP16

vhs and ICP47 genes deleted

VP16 gene with V422 mutation and insertion of IRES GFP cassette

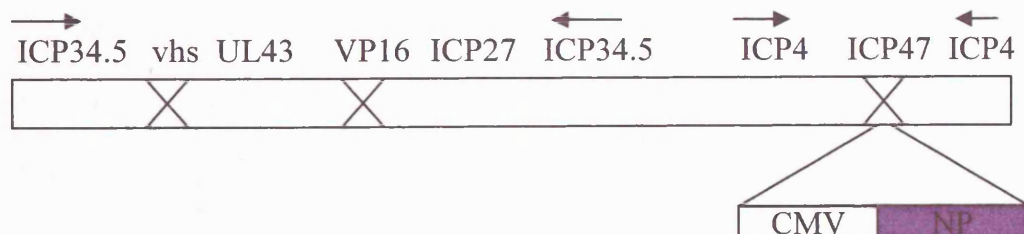


17⁺vhs⁻NP/47VP16⁻

vhs gene deleted

ICP47 gene inactivated by insertion of the CMVNP cassette

VP16 gene with V422 mutation

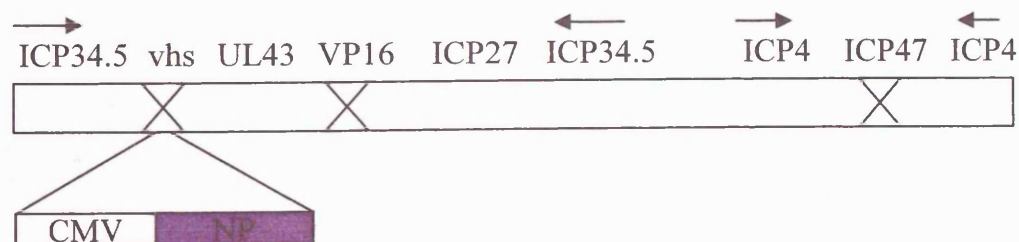


17⁺47⁻NP/vhsVP16⁻

ICP47 gene deleted

vhs gene inactivated by insertion of the CMV-NP cassette

VP16 gene with V422 mutation

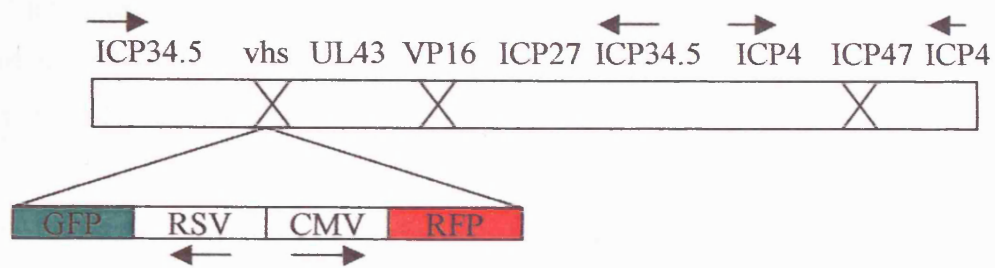


17⁺47⁻ RSVGFP-CMVRFP/vhsVP16⁻

ICP47 gene deleted

vhs gene inactivated by insertion of the RSVGFP-CMVRFP cassette

VP16 gene with V422 mutation

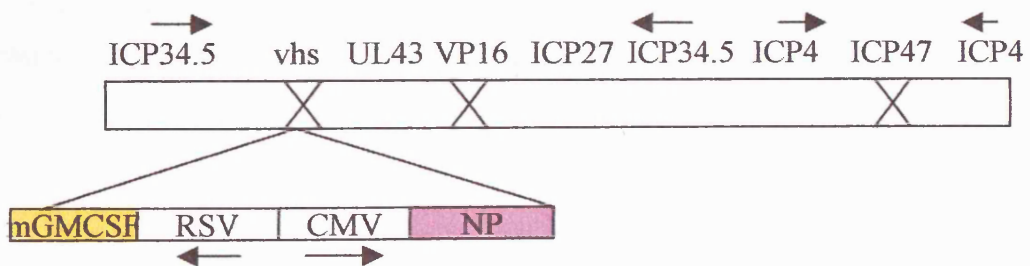


17⁺47⁻ mGMCSF-NP/vhsVP16⁻

ICP47 gene deleted

vhs gene inactivated by insertion mutation of the mGMCSF-NP cassette

VP16 gene with V422 mutation



5.2 Ability of the 17⁺vhs⁻NP/47 VP16⁻ virus to stimulate an immune response

5.2.1 Construction of 17⁺vhs⁻NP/47 VP16⁻

The 17⁺vhs⁻47⁻VP16⁻ backbone seemed, from the previous results, to be the most appropriate immunotherapeutic vector for DC based therapy. In order to determine its ability to stimulate an immune response, it was necessary to construct a virus with this backbone but expressing an immunostimulatory antigen: influenza nucleoprotein (NP) from H3N2 strain. To ensure disruption of the ICP47 gene, the NP gene was inserted at this position although insertion into vhs would have been equally appropriate. The only possible difference would have been on the efficiency of expression of NP from the different sites.

The NP gene was initially excised from pBs43NP with EcoRI and EcoRV, a plasmid already present in the laboratory based on pBluescript (pBs) and containing the HSV1 UL43 flanking regions. NP was then inserted into the pcDNA3 plasmid between the EcoRI and EcoRV sites (following the CMV promoter) forming pcDNA3NP (figure 5.1). The CMVNPpolyA fragment was purified from pcDNA3NP by digestion with NruI and BbsI and inserted at the HindIII site (blunted and dephosphorylated) of pBs47 (a pBluescript based plasmid containing the HSV1 ICP47 flanking regions), forming pCMVNP/47 (figure 5.1). After checking the integrity of the NP gene by sequencing, the pCMVNP/47 plasmid was transfected into BHK cells together with 17⁺vhs⁻CMVGFP/47 VP16⁻ viral DNA (already present in the laboratory) to allow recombination at the ICP47 locus and formation of the 17⁺vhs⁻NP/47 VP16⁻ virus. White plaques were purified.

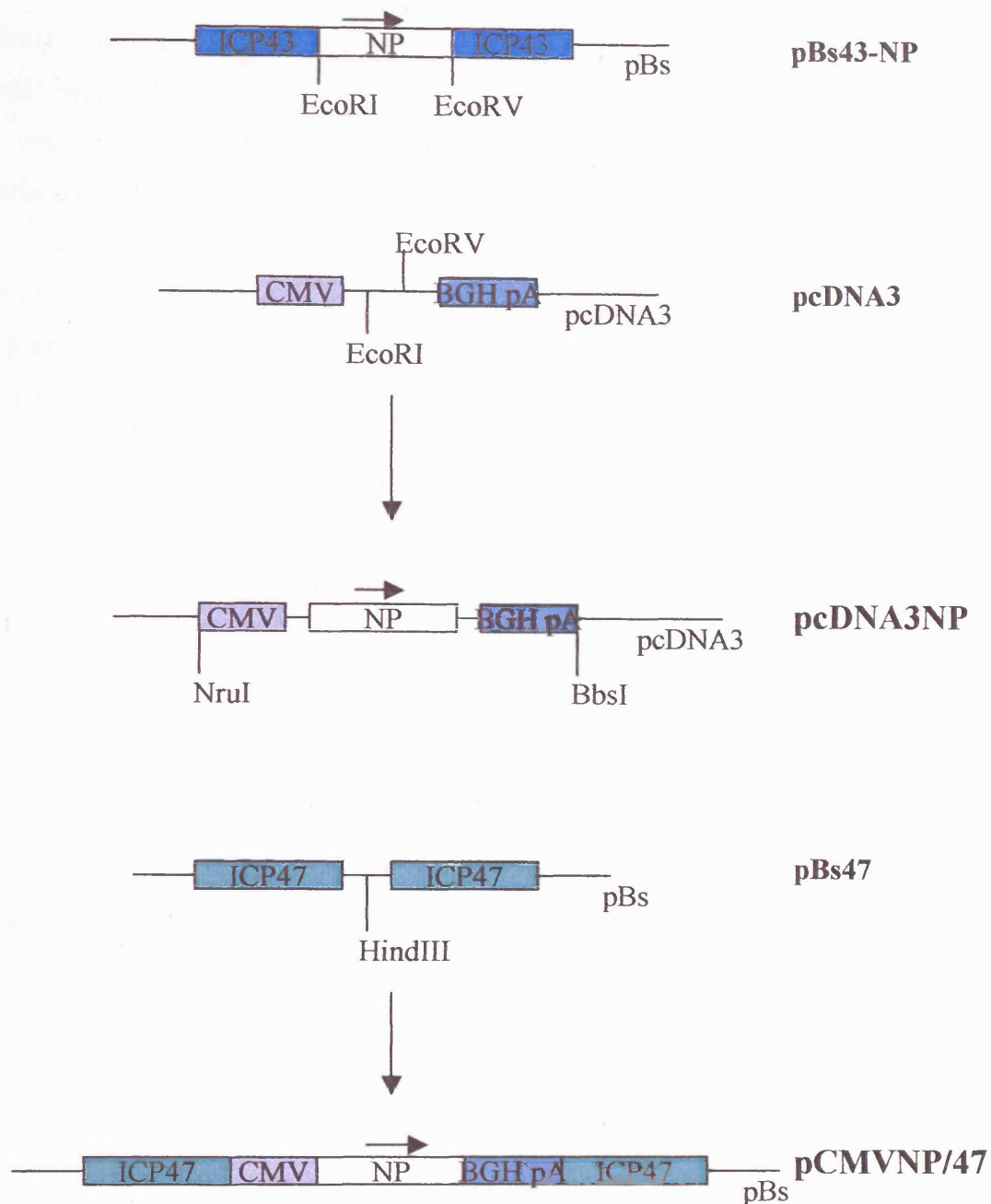


Figure 5.1: Construction of pCMVNP/47

Cloning strategy for the construction of the plasmid encoding the NP gene driven by the CMV promoter between the ICP47 flanking regions of HSV1.

Three pure white plaques (17^+ vhs $^-$ NP/47 VP16 $^-$) were obtained and tested by Southern blot to ensure presence of the NP gene (figure 5.2). The viral DNAs from the purified plaques and from the 17^+ vhs $^-$ 47 $^-$ IRESGFP/VP16 $^-$ virus (negative control) as well as the pCMVNP/47 plasmid (positive control) were digested with BamHI, which cuts twice within the NP gene and at either sides (figure 5.2). The blot was probed with an NP fragment comprising the majority of the gene linearised with HindIII from pcDNA3.

As it can be seen from figure 5.2, all three plaques showed the expected ~1.3Kb, 291bp and 216bp bands, confirming the presence of the NP gene. Plaque 2 was grown as larger stock for subsequent experiments.

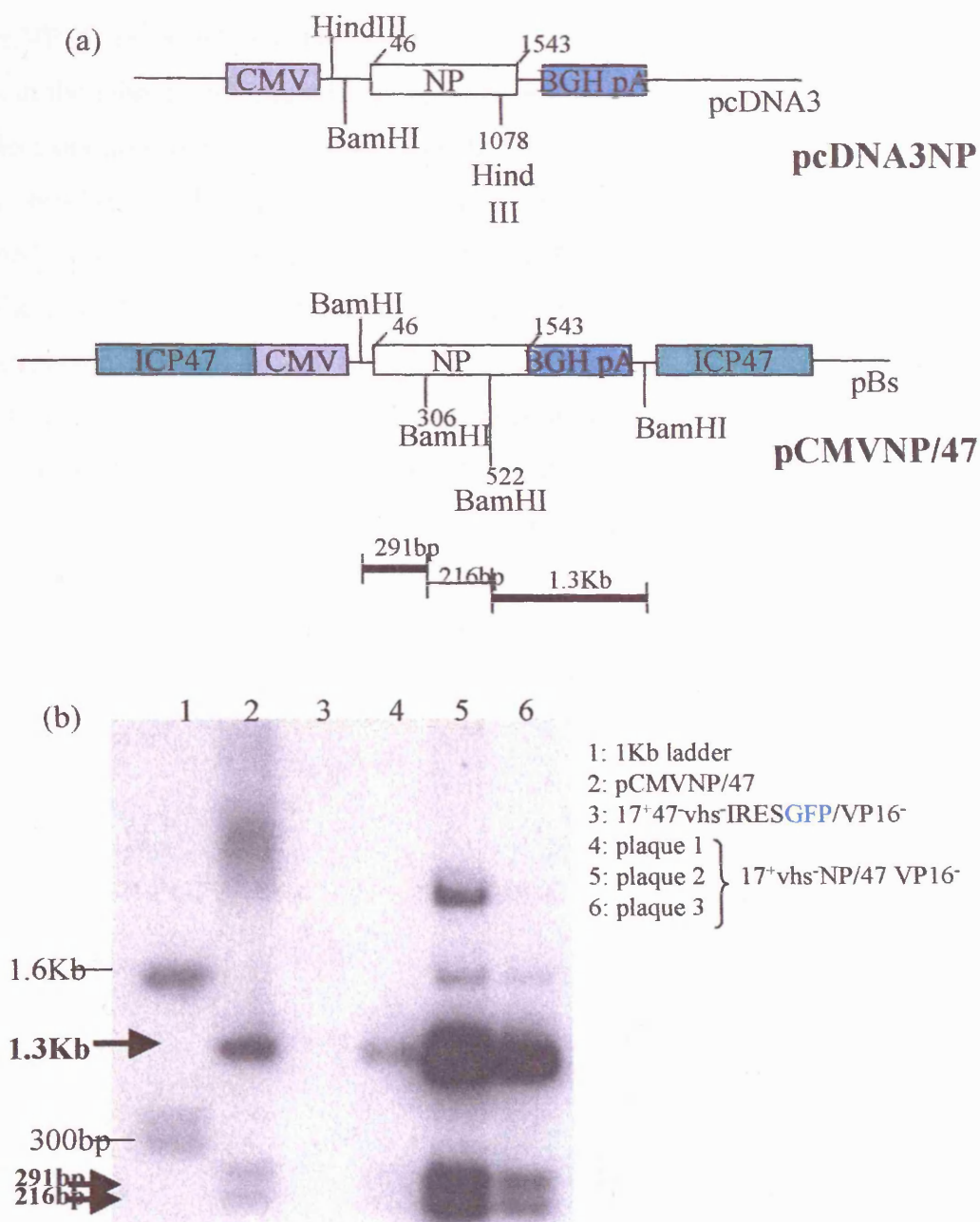


Figure 5.2: Anti-NP Southern Blot

(a) Diagram of the cassette inserted into the viral DNA showing the BamHI sites and the fragments expected.

(b) Southern blot of viral DNA from three pure plaques of 17⁺ vhs⁻ NP/47 VP16⁻ digested with BamHI and probed with NP fragment excised from pcDNA3NP using HindIII. The negative control is 17⁺ vhs⁻ 47⁻ IRES GFP/VP16⁻ DNA. The positive control is the pCMVNP/47 plasmid digested with BamHI (260bp).

Having confirmed the presence of the NP gene in the virus, expression of the protein was investigated by Western blot and compared to expression from $17^{+}47^{-}$ NP/vhsVP16⁻ in which the NP gene is inserted in the vhs locus (prepared by others in the laboratory). Samples were extracted using the urea method (§ 2.2.16) and electrophoresed on a 10% urea SDS PAGE gel given that the expected size of NP is ~60kDa. As shown in figure 5.3, a clearly positive band was successfully obtained in the lysates of cells infected with either of the viruses containing NP, regardless of the insertion position. The band appeared at a size slightly lower than expected but this is not surprising considering the denaturing conditions. Interestingly, expression from the vhs site appeared to be slightly better than from ICP47 although quantification/equalisation of samples was not carried out. Despite this, judging from the background bands, the two samples appear to have been loaded in similar amounts. Because of this comparison, and because in mouse cells it is essential to inactivate vhs whereas ICP47 does not have much activity (Ahn et al., 1996), it was decided to use the $17^{+}47^{-}$ NP/vhsVP16⁻ virus for further experiments.

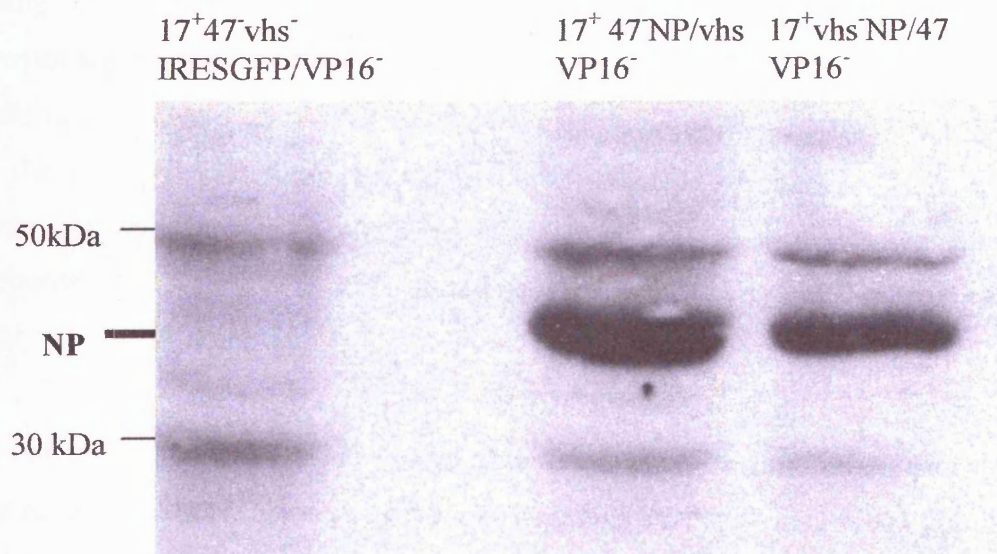


Figure 5.3: Anti-NP Western blot comparing vhs and ICP47 as insertion sites

10% Urea SDS PAGE gel stained for influenza NP. Cells were infected with virus not containing NP ($17^{+}47^{-}$ vhs⁻IRESGFP/VP16⁻) or with NP inserted in either vhs (17^{+} 47NP/vhs VP16⁻), or inserted in ICP47 (17^{+} vhs⁻NP/47 VP16⁻).

5.2.2 IFN γ response induced by 17⁺47⁻NP/vhsVP16⁻ at 1 month post-immunisation.

5.2.2.1 IFN γ response to NP

To obtain the strongest immune response, it is important to optimise the route and dose of immunisation. Initially it was decided to look at the immune response induced by 5×10^5 pfu or cells one month after immunisation since this was the dose used in the previous migration experiments. Two (figure 5.4 a) or three (figure 5.4 b) mice per group were immunised in one footpad with either 17⁺47⁻NP/vhsVP16⁻ (V), DCs infected at MOI1 with 17⁺47⁻NP/vhsVP16⁻ (DCV), mature DCs loaded with NP CD8⁺ peptide (DCNP), or, as negative control, mice received non pulsed mature DCs (DC) or nothing. Based on the literature (Gardner et al., 2000, Hocknell et al., 2002, Wang et al., 2002), spleens were removed one month after immunisation and the cells were cultured in vitro for 7 days in medium containing 10 μ M NP peptide. The IFN γ response produced by immunisation was then measured with an IFN γ ELISPOT assay. No immune response could be detected from this experiment (not shown), it was therefore decided to repeat the experiment but increasing the dose of immunogen, thus using 10^6 cells or pfu and immunising both in footpad and flank. As for the previous experiment, spleens were removed one month after immunisation and cells restimulated 7 days in vitro with NP peptide before setting up the ELISPOT. In the assay, T cells were incubated either with naïve stimulators (A20) or with stimulator cells presenting the NP peptide (A20NP). To prove antigen specific response, the responder T cells should only be activated by A20NP, resulting in IFN γ release from the T cells.

To ensure that the T cells are indeed able to release IFN γ , and therefore confirm any negative results, responders from each group were also incubated with PMA and ionomycin and all showed strong IFN γ release. As it can be seen from figure 5.4, T cells from mice that had received peptide loaded DC, virus infected DCs or neat virus, all stimulated an NP specific response. Immunisation with peptide loaded DCs was the general positive control of this experiment and, as expected, it gave a strong response. Similarly, as expected, non immunised mice did not respond to presentation of the NP peptide. It can also be seen that there is large

variability between the members of a same group, especially when DCs are used for the immunisation. Nevertheless, the results obtained from non immunised mice should be compared to mice immunised with neat virus, and responses from injection of naïve DC compared to those obtained with infected DC. Interestingly, some activity, although lower, was also seen from spleen cells of mice immunised with non-pulsed DCs. This response can not be attributed to in-vitro priming since it does not take place on cells from non-immunised mice but, rather, initial activation of T cells may result in vivo due to some factor present in the DC culture medium. In vitro restimulation then possibly primes these already active cells against NP. Despite this, it can still be concluded that there is an NP specific response stimulated both by immunisation with infected DCs and neat virus, and that using infected DCs gives a stronger response than immunisation with neat virus.

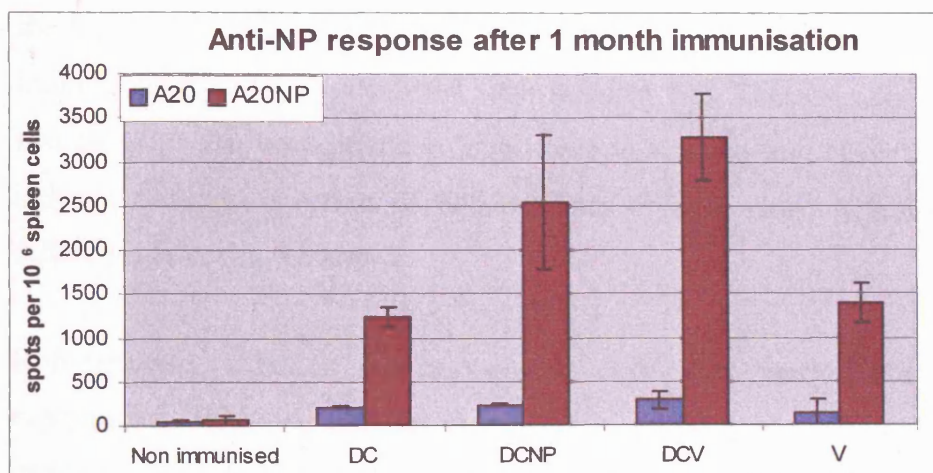


Figure 5.4: Anti-NP IFN γ ELISPOT 1 month after immunisation

IFN- γ ELISPOT measuring specific responses to NP from mice that had been immunised for 1 month in both footpad and flank with nothing (non immunised), non infected mature DC (DC), NP pulsed mature DC (DC NP), DC infected at MOI1 with 17⁺47NP/vhsVP16⁻ (DCV), or with virus alone (V). IFN γ release is measured in response to presentation of the NP peptide by A20 cells (A20NP), and background IFN γ release levels are obtained by incubation with non pulsed A20. The values shown are the average from two mice per group and the error bars are the standard deviation from triplicate values from each sample.

5.2.2.2 IFN γ response to HSV-1

In addition to looking at the response to the antigen carried by the virus, the response to the virus itself was also investigated one month after immunisation. On the day of spleen removal, an IFN γ ELISPOT using HSV infected DCs as stimulators were set up with the fresh splenocytes. DC were used rather than A20 cells since these are the cells that present the virus in vivo and, also, it is not known how well A20 cells are infected by HSV1 or how their presentation mechanism is affected by it.

As expected, following injection of virus or virally infected cells, a relatively strong IFN γ response was observed (figure 5.5). As also noticed previously, there seemed to be a specific response induced by cells of mice immunised with DC only or peptide pulsed DC, again suggesting non specific activation induced by the injection of DC. The fact that naïve DCs can activate T cells is supported by the fact that in this experiment there is some IFN γ release also from cells of non immunised mice when incubated with infected DC. These results show overall that although the background is high, there is a strong anti-HSV IFN γ response induced both by injection of infected DCs or neat virus, with no significant difference between the two.

Unfortunately, although the response to HSV was reproducible in repeated experiments, this was not the case for the anti-NP response. In the hope to increase the anti-NP response to a level that would probably give more reproducible results, it was decided to add a 1 week boost to the immunisation regime.

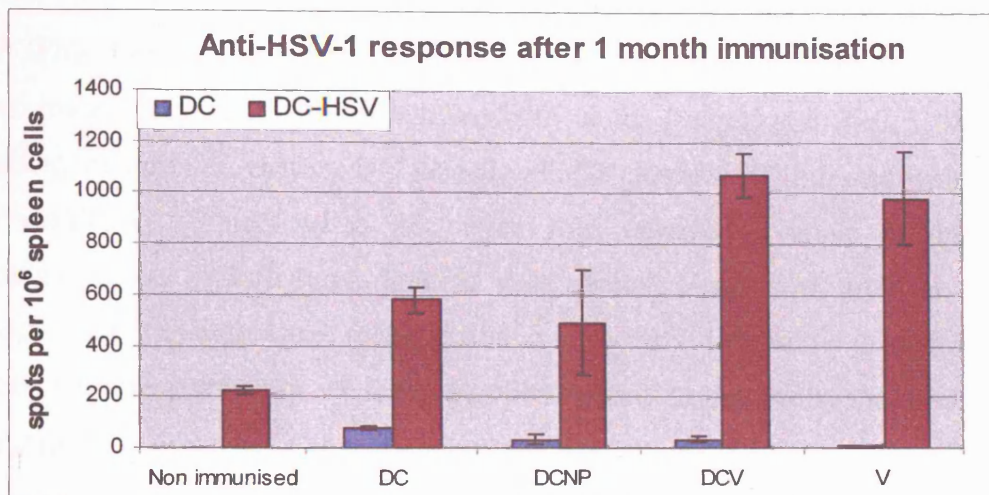


Figure 5.5: Anti-HSV1 IFN γ ELISPOT 1 month after immunisation

IFN- γ ELISPOT measuring specific antiviral response from mice immunised from 1 month in both footpad and flank with nothing (non immunised), 10^6 non infected mature DC (DC), NP pulsed mature DC (DC NP), DC infected at MOI1 with 17^+47^- NP/vhsVP16 $^-$ (DCV), or with virus alone (V). Viral antigens are presented by DCs infected with 17^+47^- NP/vhsVP16 $^-$ that act as stimulators, and background IFN γ release levels are measured by incubation with non infected DC. The values shown are the average response from two mice per group and are from one experiment representative of two. The error bars are the standard deviation from triplicate values from each sample.

5.2.3 Effect of boosting on IFN γ responses

5.2.3.1 IFN γ response to NP measured by ELISPOT

An IFN γ response has been shown to be induced over tolerance using repetitive cell stimulation and a high dose of antigen (Feunou et al., 2003, Huang & Yang, 2004, Lang & Nemazee, 2000). It was therefore thought that a more reliable anti-NP IFN γ response might be achieved after immunisation followed by boosting. Two mice per group were immunised both in the footpad and flank with either nothing, mature DC, mature DC loaded with NP peptide, DC infected with 17⁺47⁻ NP/vhsVP16⁻, or neat virus, the mice then received a boost of the same immunogen one month later. Spleens were removed one week after the second injection and the cells were restimulated in vitro for 7 days in the presence of NP peptide before setting up an IFN γ ELISPOT assay to determine the presence of anti-NP IFN γ secreting cells. As predicted, introducing a boosting immunisation improved the reproducibility of the results. Figure 5.6 shows the results obtained from one experiment, representative of two, and shows the average number of spots obtained from three mice per group. As before, the very few spots appearing in wells from spleen cells of non immunised mice suggest that the response seen upon neat virus injection is dependent on NP expression. In addition, the response also appeared to be NP specific since very few cells reacted to A20 cells alone. Interestingly, the intensity of the response obtained following immunisation with neat virus was similar to that obtained without boosting (~1200 spots), implying that boosting does not have an amplifying effect. Immunisations involving DCs still presented background problems, and infected DCs seemed to give similar number of IFN γ producing cells as the group immunised with naïve DCs. As previously suggested, T cells from mice immunised with DCs only are probably already activated (possibly by the FCS) and undergo further activation upon incubation with antigen presenting cells (A20NP). Non specific activity of T cells after DC injection seems to be higher following boost than after single immunisation, but the response stimulated by infected DCs, disappointingly, did not appear to be higher than background, suggesting abolition of the antigen response by boosting.

The results presented, therefore, allow the conclusion that immunisation with neat virus can successfully stimulate an NP specific CD8⁺ IFN γ response and that this can be obtained more reliably if a boosting step is added. Boosting of infected cells, on the other hand, abolished the response, suggesting that expression of a high amount of NP is beneficial but should be achieved by means other than boosting.

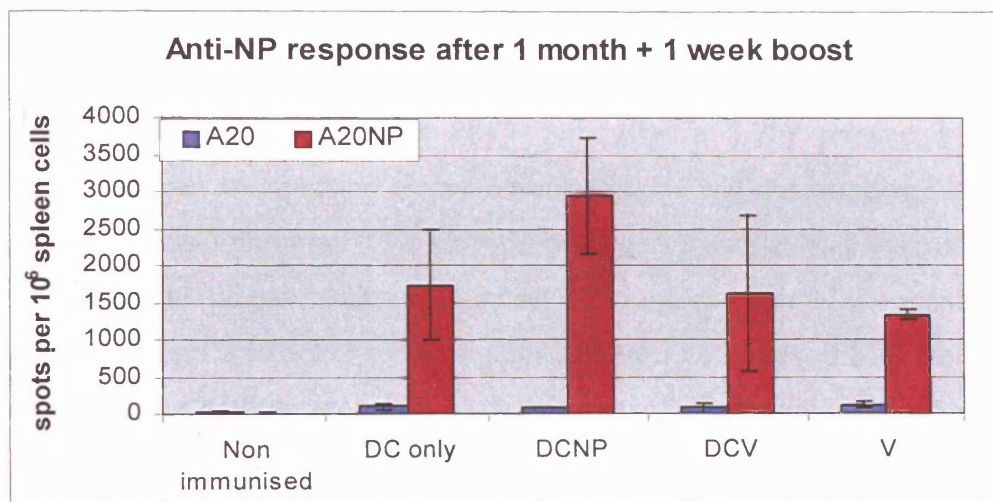


Figure 5.6: Anti-NP IFN γ ELISPOT 1 month + 1 week boost after immunisation

IFN γ ELISPOT to measure response to NP from mice immunised for 1 month and boosted for a week with nothing (non immunised), 10⁶ mature non infected DC (DC), 10⁶ mature NP pulsed DC (DCNP), 10⁶ DC infected at MOI 1 with 17⁺47NP/vhsVP16⁻ (DCV), or with the virus only (V). The values shown are from one experiment representative of two and are the average response from three mice per group. Responder T cells were incubated with NP peptide pulsed A20 cells (A20NP), or naïve A20 cells (A20).

5.2.3.2 CTL response to NP measured by ^{51}Cr release assay

In addition to the IFN γ ELISPOT assay for the determination of the anti-NP response, a ^{51}Cr release assay was carried out to assess cytotoxicity of the activated T cells (figure 5.7). The splenocytes from three immunised mice that received a boost one month after the initial immunisation were restimulated in vitro for 7 days in presence of NP peptide and irradiated spleen cells (§ 2.4.5), and were used to set up the assay at various effector:target ratios. T cells were incubated either with ^{51}Cr loaded P815 cells alone or with ^{51}Cr loaded P815 presenting the NP peptide. Figure 5.7 shows the average value obtained from the three mice per group. Figure 5.7a indicates that there is a relatively high degree of lysis of P815 cells including when these are not presenting the NP peptide. This suggests that the T cells are non specifically activated, and are therefore able to lyse cells regardless of peptide presentation. Interestingly, the results obtained from incubation with NP pulsed P815 cells (figure 5.7b) showed a clear distinction between the reaction from immunised mice and the negative controls (DC only and non immunised) which would suggest specific lysis even though this is not actually the case. Mice immunised with peptide loaded DCs or infected cells do not present a stronger reaction than that seen with naïve P815, therefore indicating that the activity seen is not specific to the NP peptide but due to non-specific activation of the cells. The results obtained from mice immunised with neat virus, on the other hand, show an increased reactivity upon incubation with peptide presenting P815, thus showing that these cells can specifically recognise and lyse cells presenting the NP peptide.

Overall, as for the ELISPOT results, no specific anti-NP reaction could be seen after immunisation with infected DCs, but despite the strong over-activation of the splenocytes, it was still possible to identify an NP specific response from cells of mice immunised with neat virus. In conclusion, it is possible to say that the T cells, of boosted mice are highly active, able to lyse A20 cells non-specifically, and only those from mice immunised with neat virus are able to lyse cells at a higher level than background. This was also noticed in the ELISPOT assay, showing specificity of response.

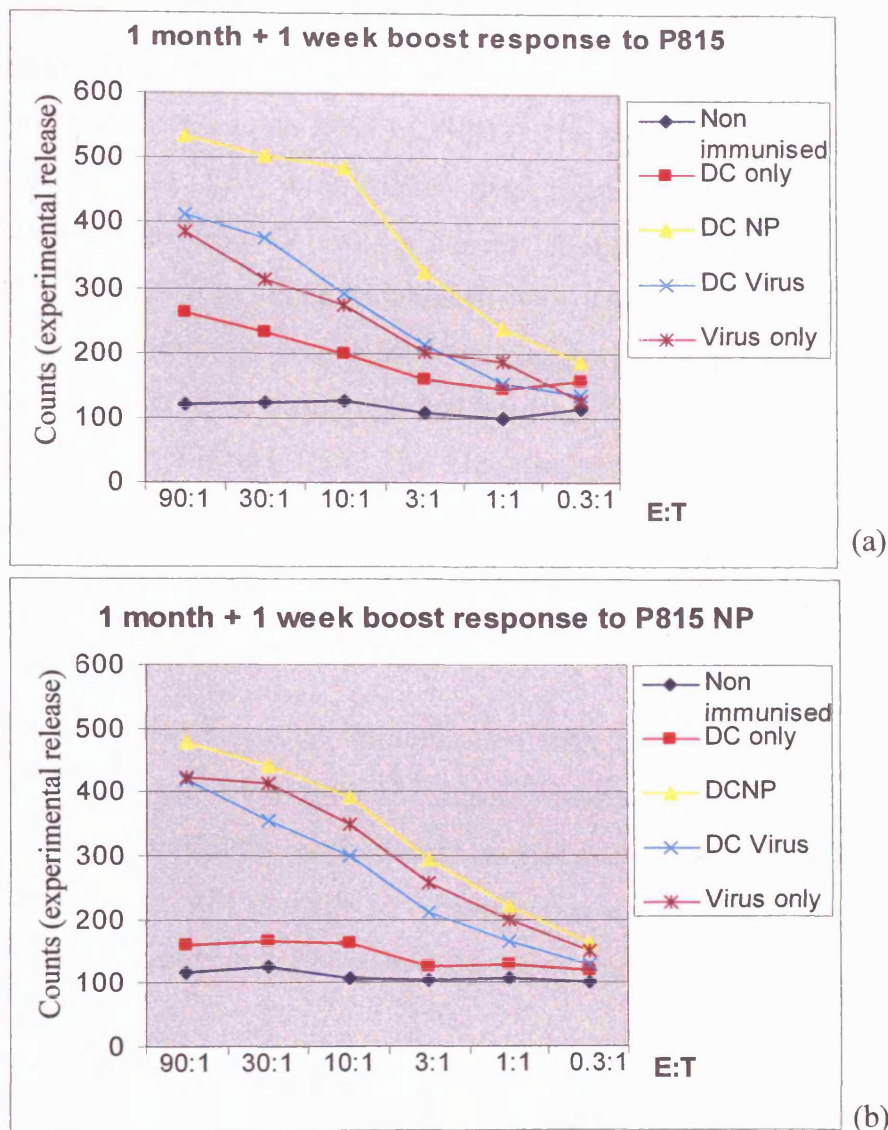


Figure 5.7: ^{51}Cr release assay anti-NP at 1 month + 1 weeks boosting

^{51}Cr release assay after immunisation for 1 month + 1 week boosting with nothing (non immunised), 10^6 mature non infected DC (DC only), mature DC that have taken up NP peptide (DCNP), DC infected at MOI 1 with $17^{+47}\text{NP}/\text{vhsVP16}^{-}$ (DC Virus), or with 10^6 pfu of $17^{+47}\text{NP}/\text{vhsVP16}^{-}$ only (V only). The values shown are the average of data from three mice. T cells were incubated with naïve P815 cells (a) or peptide pulsed P815 cells (b).

5.2.3.3 IFN γ response to HSV1

As for the previous experiment, in addition to the anti-NP response, the antiviral IFN γ response was measured after one month and one week boosting immunisation. Non restimulated splenocytes were incubated with HSV1 infected DCs or uninfected DCs in an IFN γ ELISPOT. The responses seen following a 1 week boost (figure 5.8) were higher than those recorded after 1 month immunisation (figure 5.5). Comparing the results obtained from non immunised mice with the ones from mice immunised with neat virus a strong anti-viral response is seen. Similarly, despite the high background from peptide loaded DC as previously observed, a strong antiviral response can also be seen following immunisation with infected DCs. The high background makes it difficult to compare whether a stronger response is obtained after immunisation with infected DCs or neat virus, although the strong anti-HSV response from DCV might explain the lack of reaction from this group in the anti-NP ELISPOT.

Overall these results show that immunisation with neat virus clearly induces strong anti-NP and anti-viral IFN γ responses, whereas immunisation strategies that involve injection of DCs result in high background readings in the ELISPOT assay. However, it is still possible to conclude that stimulation of anti-NP and anti-HSV IFN γ responses may be occurring using the method tested.

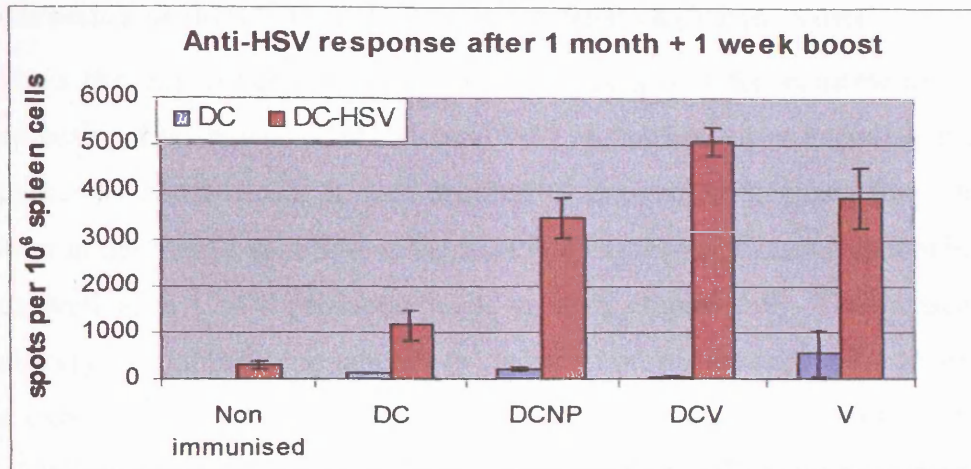


Figure 5.8: Anti-HSV1 IFN γ ELISPOT after 1 month + 1 week boost immunisation

IFN γ ELISPOT to measure the antiviral response from mice immunised for 1 month and boosted for a week with nothing (non immunised), 10^6 mature non infected DC (DC), 10^6 mature NP pulsed DC (DCNP), 10^6 DC infected at MOI 1 with 17⁺47-NP/vhsVP16⁻ (DCV), or with the virus only (V). The values shown are from one experiment representative of two independent ones, and are the average response from two mice per group. The antiviral response is given by incubation of T cells with DCs infected with 17⁺47-NP/vhs VP16⁻, whereas negative controls are the values obtained using non infected DCs as stimulators.

5.3 Effect of co-expressing GMCSF on the ability to stimulate an immune response

5.3.1 Construction of the 17⁺47⁻ RSVGFP-CMVRFP/vhs VP16⁻ virus

HSV-1 allows the expression of multiple genes. As a tool for construction of viruses expressing two genes (GMCSF and NP), a further virus encoding two marker genes was constructed. It was decided to express both genes from the same location in the virus backbone, using a double expressing cassette containing an RSV as well as a CMV promoter back to back (figure 5.9). The pvhsDC plasmid (already available in the laboratory) is based on pBluescript and contains the double expressing cassette between the vhs flanking regions, this is to allow insertion of the recombinant genes at this location of the viral genome. GFP and RFP were therefore cloned into this plasmid. GFP was purified from the hrGFP plasmid digesting with NotI and XhoI and was subsequently blunted before being inserted at the PmeI blunt site of pvhsDC, under the RSV promoter, forming pvhsDCGFP. RFP was purified from the pDsRedN1 plasmid via BamHI and NotI digestion and then blunted before cloning it into pvhsDCGFP following the CMV promoter, at the ApaI site, forming pvhsDCGFP-RFP (figure 5.9). This plasmid was transfected with the viral DNA from 17⁺vhs⁻47⁻VP16⁻ to achieve insertion at the vhs site, and plaques that resulted both green and red were purified. The purified plaques clearly appeared to express both genes under fluorescence (figure 5.10), showing that both genes had been successfully incorporated.

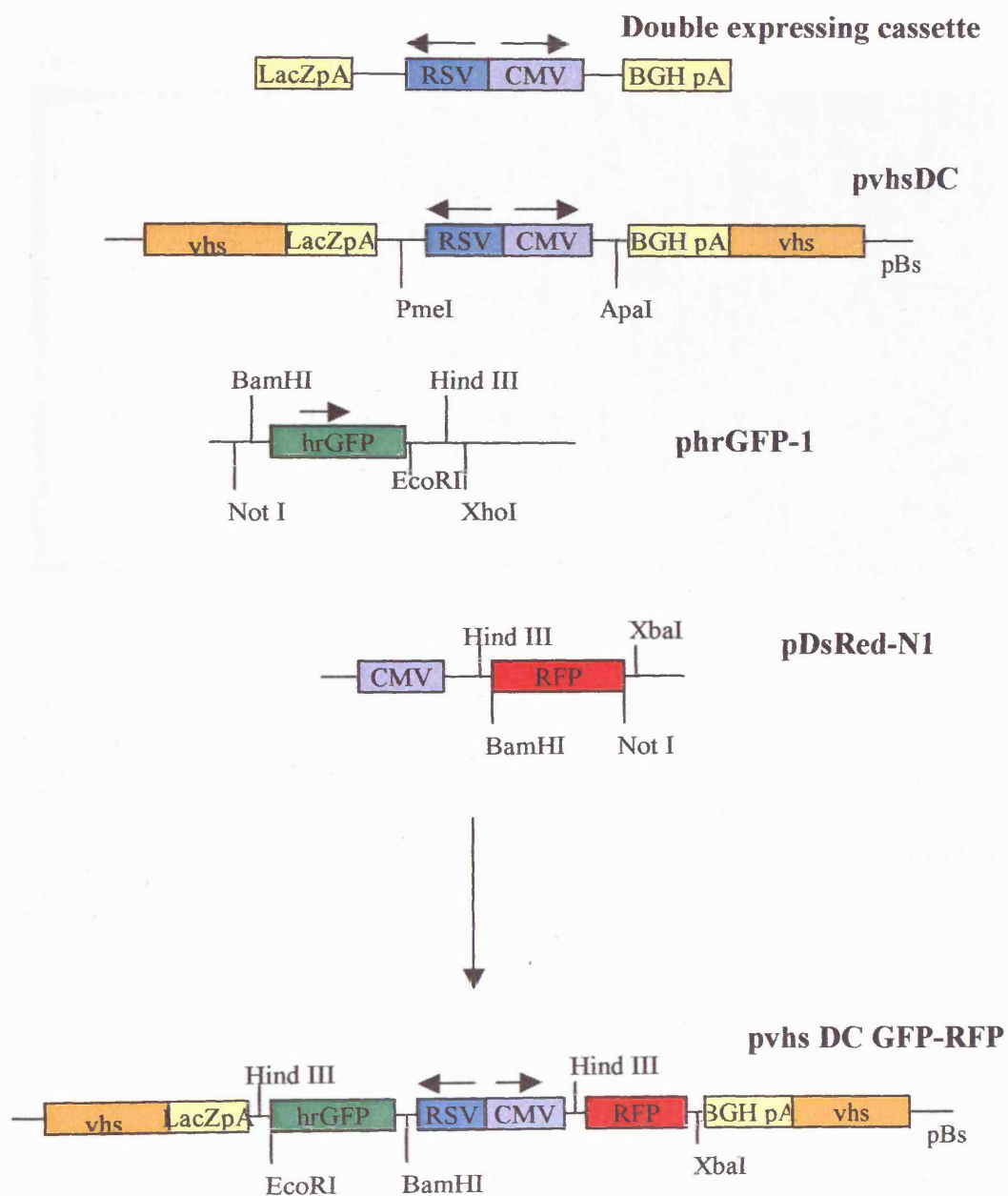


Figure 5.9: Construction of pvhsDC GFP-RFP

Cloning strategy for the insertion of the GFP and RFP genes, into the double expression plasmid containing the vhs flanking regions

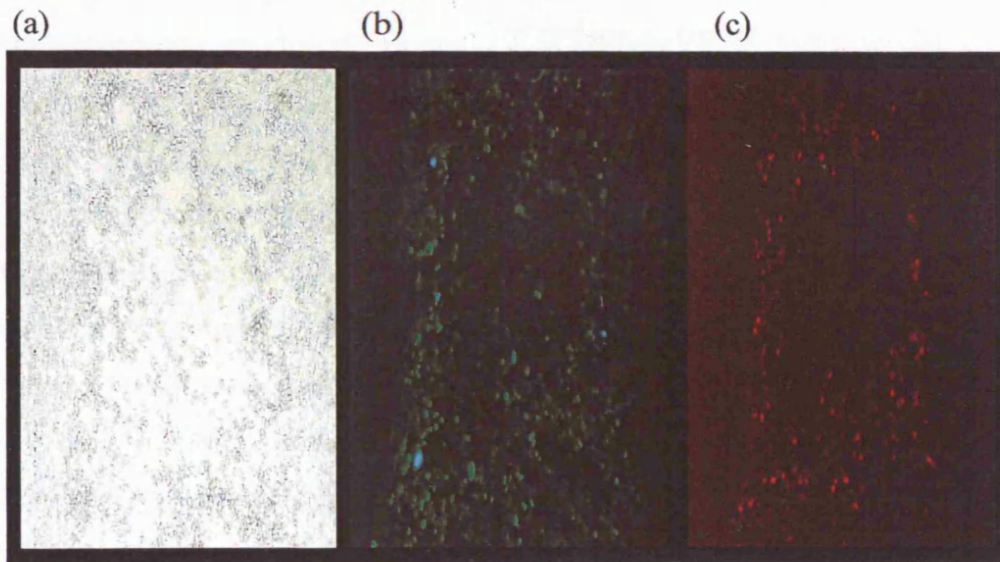


Figure 5.10: 17⁺47⁻ GFP-RFP/vhs VP16 plaque expressing both GFP and RFP genes

Plaque from BHK cells infected with 17⁺47⁻ GFP-RFP/vhs VP16 . The same plaque was photographed under the light microscope (a), showing the infected rounded cells, and using different fluorescence filters to show expression of both GFP (b) and RFP (c) in the infected cells.

5.3.2 Construction of the 17⁺47⁻mGMCSF-NP/vhs VP16⁻ virus

Taking into account previous reports (Bukreyev et al., 2002, Lu et al., 2002, Rodriguez et al., 1999, Wang et al., 2002, Weiss et al., 1998), it seemed possible that immune responses produced by the 17⁺47⁻NP/vhsVP16⁻ virus would be increased by co-expressing murine GMCSF (mGMCSF). A virus with the same structure as the 17⁺47⁻RFP-GFP/vhsVP16⁻ was therefore constructed expressing mGMCSF from the RSV promoter and NP under the CMV promoter (figure 5.11). Both genes were cloned into pvhs DC, initially GMCSF was purified from pGemTeasyGMCSF (provided by other members of the laboratory) with NotI and blunted before being inserted at the PmeI site of pvhsDC to form pvhsDCGMCSF. As a second step NP was isolated from pcDNA3NP (§ Figure 5.1) by initially linearising the plasmid with KpnI followed by blunting and then digestion with ApaI. The purified NP gene was inserted between the PmlI and the ApaI sites of pvhsDCGMCSF forming the pvhsDCGMCSF-NP plasmid (figure 5.11).

To make the 17⁺47⁻GMCSF-NP/vhsVP16⁻ virus, pvhsDCGMCSF-NP was transfected into BHK cells with the viral DNA of 17⁺47⁻RFP-GFP/vhsVP16⁻ to allow for recombination at the vhs site. Because it is possible for recombination to take place between one flanking region and the linker sequence between the two promoters, to ensure that both genes would be incorporated in the virus it was important to check that the purified plaques were neither green nor red.

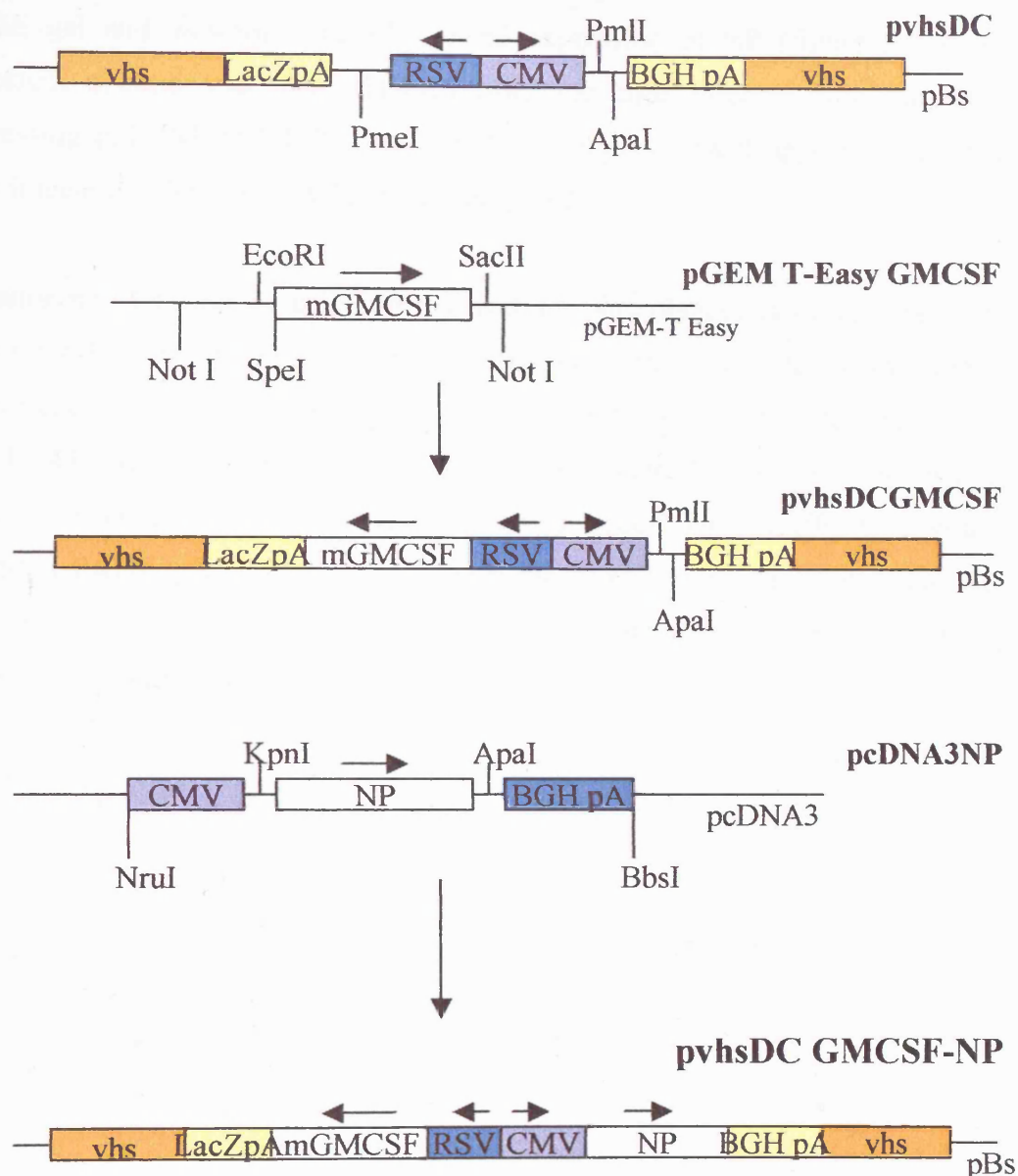


Figure 5.11: Construction of pvhsDC GMCSF-NP

Cloning strategy for the construction of the plasmid expressing both GMCSF and NP from the double expressing cassette inserted between the vhs flanking regions. In the first step of cloning GMCSF was inserted following the RSV promoter and subsequently NP was inserted at a position following the CMV promoter.

Four pure plaques were obtained and tested both for NP and for mGMCSF expression. Cell lysate samples from the purified plaques, run on a 10% urea SDS PAGE gel and western blot, all showed expression of NP (figure 5.12). As expected, a band was also obtained from the cells infected with the virus expressing only NP (17⁺47NP/vhs VP16⁻) whereas no band appeared from the cells infected with the RFP-GFP expressing virus.

The supernatants from the cells infected with the pure plaques as well as that from cells infected with the RFP-GFP virus (negative control) were tested by ELISA for presence of GMCSF. It appeared (figure 5.13) that infection with either of the four 17⁺47⁻GMCSF-NP/vhsVP16⁻ pure plaques produced significant amounts of GMCSF, whereas this was not the case from infection with the RFP-GFP virus. The SDS PAGE gel results together with the ELISA results indicated that the virus was properly constructed, able to express both the NP and the GMCSF genes in non complementing cell lines.

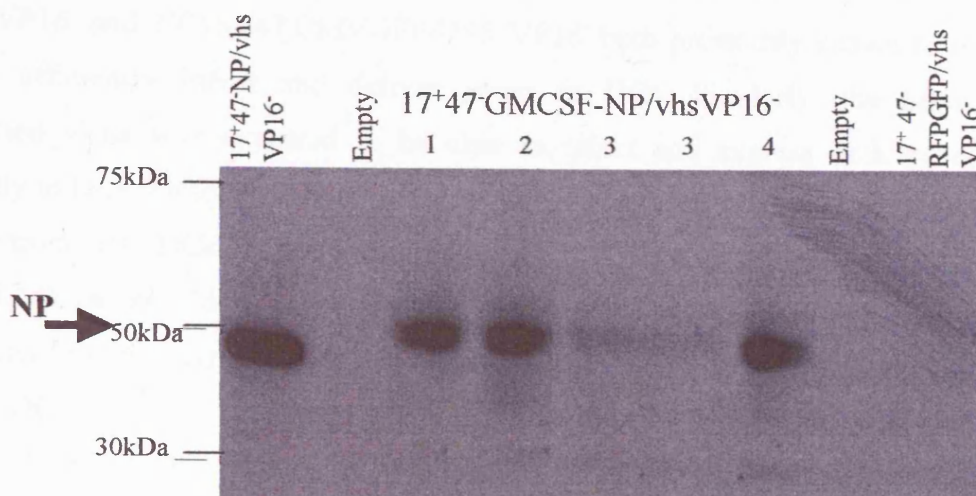


Figure 5.12: Anti-NP Western blot of extracts from 17⁺47-GMCSF-NP/vhs VP16⁻ infected BHKs

10% urea SDS PAGE gel western blotter and tested for NP expression in cell lysates of BHKs cells infected with four different pure plaques of 17⁺47-GMCSF-NP/vhsVP16⁻ (1-4), with 17⁺47-NP/vhsVP16⁻ as positive control or with 17⁺47-RFP-GFP/vhsVP16⁻ as negative control.

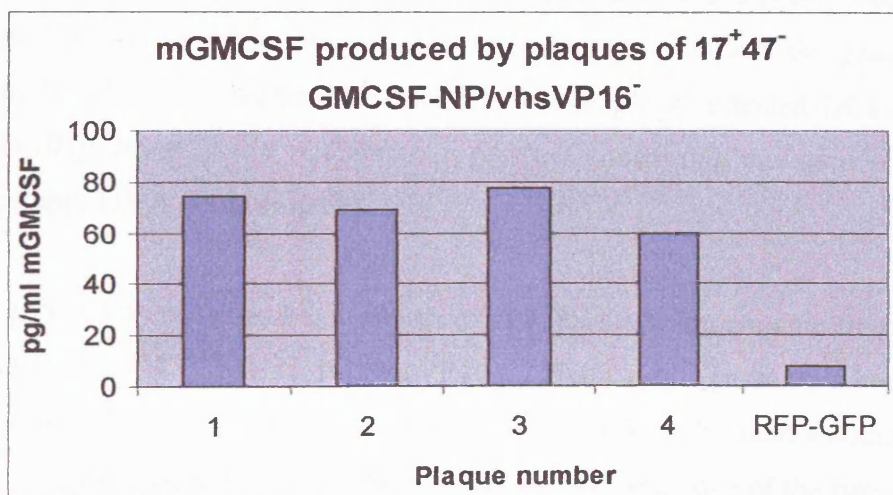


Figure 5.13: Production of GMCSF in BHKs from 17⁺47-GMCSF-NP/vhs VP16⁻ plaques

ELISA measuring GMCSF present in supernatant of BHKs cells infected with different plaques of 17⁺47-GMCSF-NP/vhsVP16⁻. Negative control is the supernatant from cells infected with 17⁺47-RFP-GFP/vhsVP16⁻ which does not express GMCSF.

5.3.3 Characterisation of 17⁺47⁻GMCSF-NP/vhsVP16⁻ infection of DC

The 17⁺47⁻GMCSF-NP/vhsVP16⁻ virus has the same backbone as the 17⁺47⁻NP/vhsVP16⁻ and 17⁺vhs⁻47⁻CMVGFP/US5 VP16⁻ both previously shown to be able to efficiently infect and deliver genes to DCs. Similarly, the newly constructed virus was expected to be able to infect and express both genes efficiently in DCs. Unfortunately, it was not possible to detect NP by Western blot starting from 10⁶ DCs infected at MOI=1 with 17⁺47⁻GMCSF-NP/vhsVP16⁻ (figure 5.14). In case this was because the amount of protein was not sufficient to be detected by western blot, RT-PCR was used. NP RNA expression from infected DCs was confirmed (figure 5.15). RNA isolated from 10⁶ non-infected DC or 10⁶ DCs infected at MOI1 with 17⁺47⁻GMCSF-NP/vhsVP16⁻ was reverse transcribed, and PCR for NP was carried out on these samples (RT PCR) using primers internal to the gene (§ 2.2.11), amplifying a 320 bp fragment (bp350-bp671). To ensure that any amplified band was not due to genomic DNA contamination, the PCR was also carried out on the same amount of non reverse transcribed RNA samples (RNA PCR). In the PCR carried out on cDNA templates, the 320bp fragment could only be amplified from the plasmid positive control and the sample from infected DCs, showing expression of the gene from infected cells. The non-reverse transcribed RNA sample of infected DCs (RNA PCR-DCGMNP sample) did not generate product confirming the sample to be free of genomic DNA contamination.

Unfortunately it was not possible to detect mGMCSF in the supernatant from DCs infected with 17⁺47⁻GMCSF-NP/vhsVP16⁻. This was probably because, considering that RSV is not as strong a promoter as CMV, only small amounts are produced. Despite this, there is no reason to believe that only one of the two genes would be expressed in DCs.

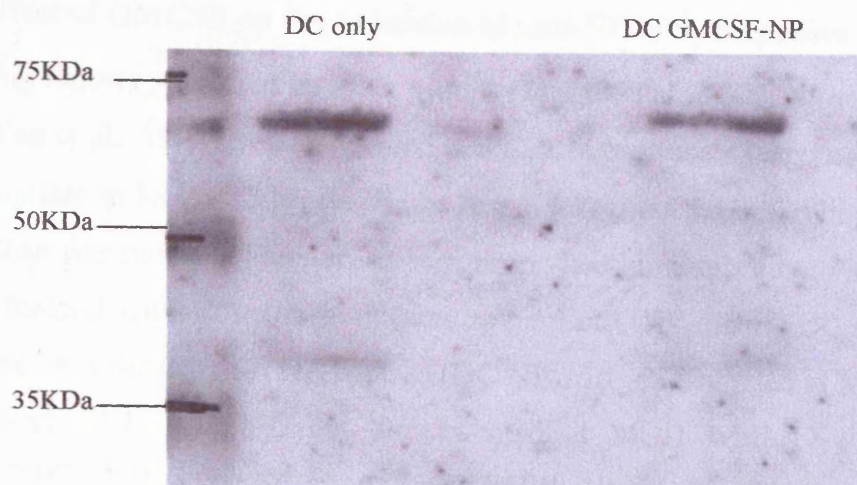


Figure 5.14: Western blot to detect NP expression in DC infected with 17⁺47-GMCSF-NP/vhs VP16⁻

Anti-NP 10% urea SDS PAGE gel on samples prepared from 10⁶ non infected DC (DC only, negative control) and 10⁶ DC infected at MOI of 1 with 17⁺47-GMCSF-NP/vhs VP16⁻ (DC GMCSF-NP), western blotted and probed with an antibody against NP.

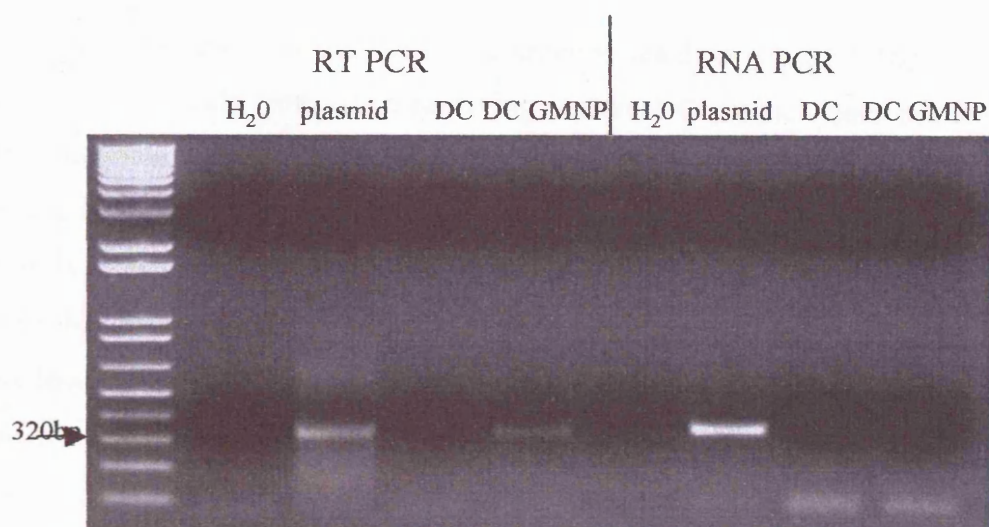


Figure 5.15: Detection of NP expression in DC by RT PCR

RT PCR: PCR carried on cDNA reverse transcribed from RNA of uninfected DC (DC) and from DCs infected MOI1 with 17⁺47-GMCSF-NP/vhs VP16⁻ (DC GMNP).

RNA PCR: these are negative controls, the PCR was carried out on total RNA isolated from the DC samples without reverse transcription.

Both PCRs were also carried out on a water negative control (H₂O), and on the pcDNA3NP plasmid that contains the NP gene, as positive control. The primers amplify a 320bp fragment (bp350-bp671).

5.3.4 Effect of GMCSF on the induction of anti-NP IFN γ responses

Following reports published by other groups (Matsui et al., 2002, Nakamura et al., 2002, Wan et al., 1999), it was decided that to assess primary responses, it would be appropriate to look at IFN γ responses two weeks after the initial immunisation (rather than one month). Three mice per group were immunised both in the flank and the footpad with either nothing (non immunised), 10^6 mature DC (DC only), 10^6 mature DCs pulsed with NP peptide (DC NP), 10^6 DC infected at MOI 1 with 17⁺47NP/vhsVP16⁻ (DCV), 10^6 DC infected at MOI1 with 17⁺47⁻GMCSF-NP/vhsVP16⁻ (DCVGM), 10^6 pfu of 17⁺47NP/vhsVP16⁻ (V) or 10^6 pfu of 17⁺47⁻GMCSF-NP/vhsVP16⁻ (VGM) and spleens were removed 2 weeks post-immunisation. Splenocytes were restimulated in vitro for 7 days in the presence of NP peptide and were then used to set up an IFN γ ELISPOT with, as stimulator, either A20 cells only or A20 cells pulsed with 10 μ M NP.

This immunisation, as with previous experiments, resulted (figure 5.16) in very high background levels from mice receiving mature DCs, which does not allow definite conclusions to be drawn as to the effect of immunisation with infected DCs. There is a strong response induced by immunisation with peptide pulsed DC that appears considerably above background, and immunising with DCs infected with the GMCSF expressing virus (DCVGM) results in all three mice giving a response lower than DCNP, but higher than the background (DC only). This is not the case for cells from mice receiving cells infected with virus not expressing GMCSF where no response above background is seen. From the analysis of these results it therefore appears that GMCSF confers an advantage to the injection of infected cells.

In order to try to compare the different groups more accurately, the values resulting from incubation of cells with A20 only (very similar for all groups) has been subtracted from the value obtained from incubation of the cells with A20NP, to give a net value (figure 5.16b). Comparison of the net values clearly show that immunisation with DCs infected with GMCSF containing virus is more efficient than when GMCSF is not present, but unfortunately because of the high

background is not possible to compare injection of DCs with injection of neat virus.

Virus injection gave a specific response not seen in non immunised mice, but addition of GMCSF did not appear to confer an advantage to the strength of the response (figure 5.16b). Looking at the overall results showing the average response obtained from the three mice (figure 5.17), the conclusion that expression of GMCSF confers an advantage to the injection of infected DCs but not to neat virus injection can be drawn.

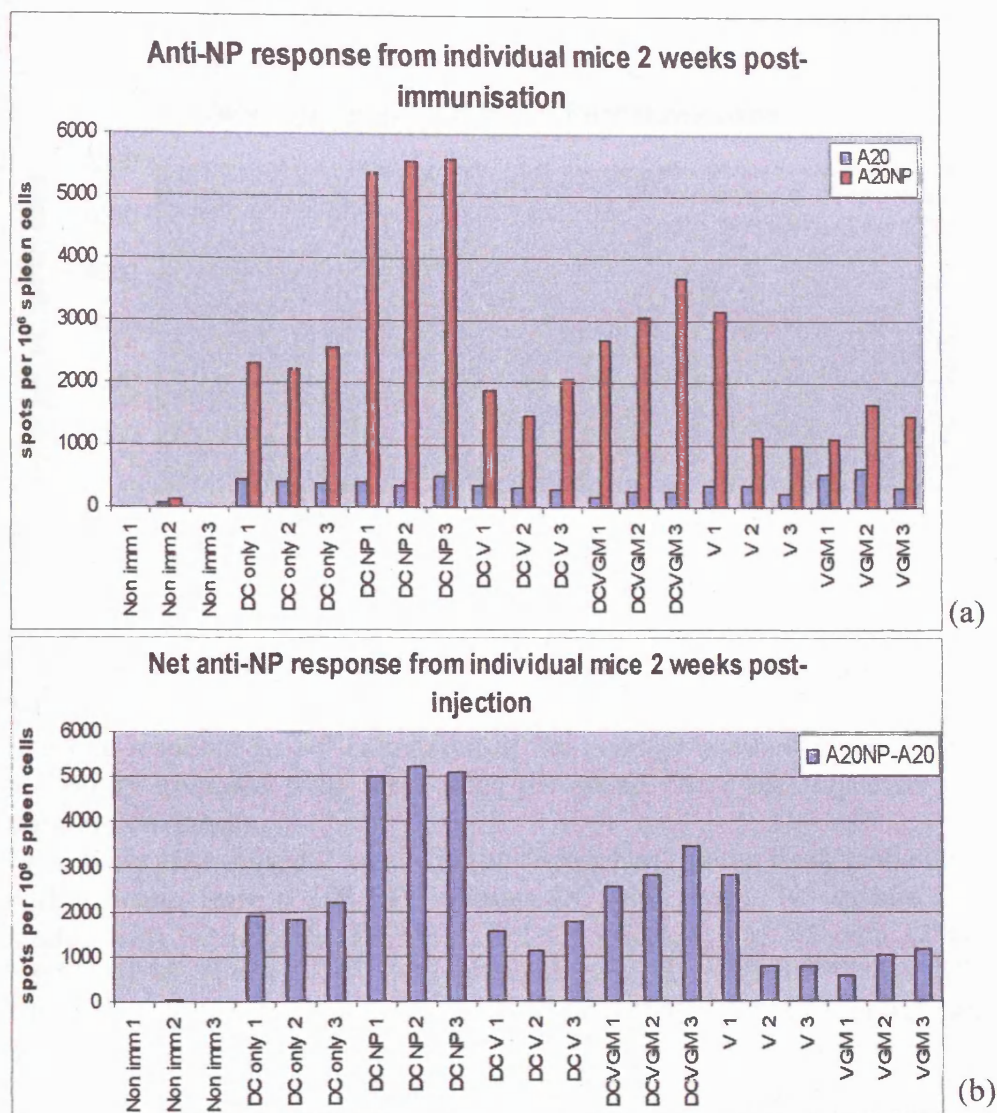


Figure 5.16: Effect of GMCSF on anti-NP immune responses

Specific immune responses to NP from individual mice as determined by IFN γ ELISPOT after two weeks immunisation in footpad and flank with nothing (non immunised), mature DC (DC only), mature DC pulsed with NP peptide (DC NP), DC infected at MOI of 1 with 17⁺47-NP/vhsVP16⁻ (DCVNP), or infected at MOI of 1 with 17⁺47-GMCSF-NP/vhsVP16⁻ (DCVGMNP), 10⁶ pfu of 17⁺47-NP/vhs VP16⁻ or 10⁶ pfu of 17⁺47-GMCSF-NP/vhsVP16⁻. (a) specific response to A20 cells only (A20) and to A20 cells presenting peptide (A20NP). (b) net response: number of spots from incubation with A20NP - number of spots from incubation with A20 only (A20NP-A20).

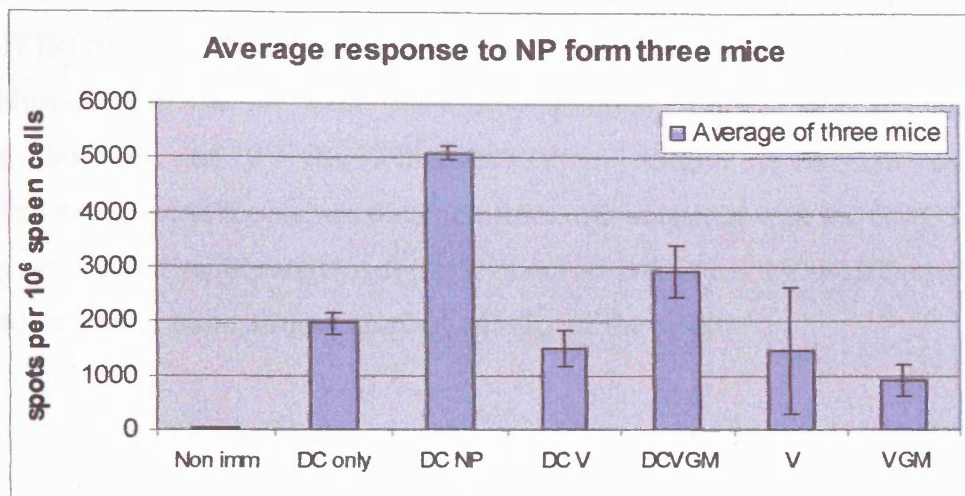


Figure 5.17: Effect of GMCSF on the average immune response to NP

Immune response to NP calculated as the average number of spots from net values obtained from three mice per group. Error bars represent the standard deviation.

Mice were immunised 2 weeks in the footpad and in the flank with either nothing (non imm.), 10^6 LPS mature DC (DC only), 10^6 mature DC loaded with NP peptide (DCNP), 10^6 DCs infected at MOI1 with 17⁺47-NP/vhsVP16⁻ (DCV), 10^6 DCs infected at MOI1 with 17⁺47-GMCSF-NP/vhsVP16⁻ (DCVGM), 10^6 pfu of 17⁺47-NP/vhsVP16⁻ (V), or 10^6 pfu of 17⁺47-GMCSF-NP/vhsVP16⁻ (VGM)

To ensure that the immune response seen following virus injection was actually due to expression of NP and not to activation of the cells from injection of virus (as is the case for DCs), the response from mice immunised with the neat GMCSF NP virus was compared to that induced by a virus not containing the NP gene ($17^+vhs^-47IRESGFP/VP16^-$). It appeared that the response seen was specifically due to expression of the NP gene since only minimal response was mounted following injection of $17^+vhs^-47IRESGFP/VP16^-$ (figure 5.18). In this independent experiment, the values obtained from immunisation with the GMCSF NP virus were in the same range as the one obtained previously (400-1300 spots per 10^6 spleen cells), indicating the reproducibility of the result.

The $17^+47^-GMCSF-NP/vhsVP16^-$ virus is therefore able to stimulate an $IFN\gamma$ response against the recombinant protein, as can be seen from the neat virus injection groups. However, addition of GMCSF does not seem to confer an advantage by improving this response. On the other hand, co-expression of GMCSF may have a beneficial effect in improving the response stimulated following the injection of infected DCs.

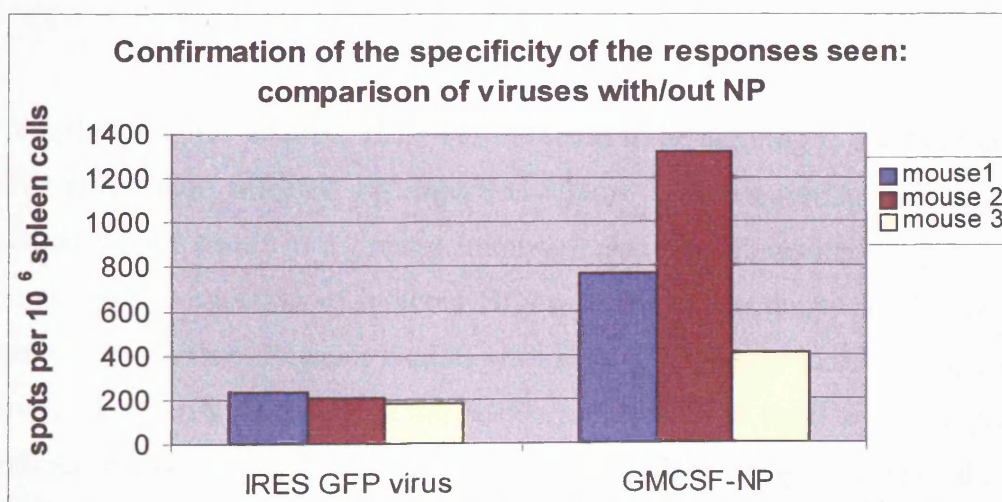


Figure 5.18: Specificity of immune responses following injection of neat virus

Anti-NP $IFN\gamma$ response from mice immunised with viruses expressing or not expressing the NP gene. Three mice were immunised 2 weeks with either 10^6 pfu of $17^+vhs^-47IRESGFP/VP16^-$ or with 10^6 pfu of $17^+47^-GMCSF-NP/vhsVP16^-$.

5.4 Discussion

The main focus of this study was to determine whether an immune response could be induced to a recombinant antigen carried by an HSV-1 vector, and whether this would be stimulated after either injection of infected cells or neat virus. These results have shown that a 17⁺vhs⁻47⁻VP16⁻ HSV1 virus expressing NP can induce an IFN γ response to it. Replication incompetent HSV-1 viruses have previously been used as vectors to stimulate an immune response (§5.1). The results presented here show that this may also be possible by injection of transduced DCs. The mechanism by which the response is stimulated has not been investigated. IFN γ ELISPOT assays do not specify the type of cells involved: CD4⁺ Th cells, NK cells as well as IFN γ secreting cells can release IFN γ . Previous studies using HSV1 have shown that the response induced did not involve CD4⁺T cells (Lauterbach et al., 2004), while CTL and B cell responses to the antigen carried have been shown to be stimulated (Brockman & Knipe, 2002, Willis et al., 2001). Similarly, here, the ⁵¹Cr release assay indicated that CTL cells play a part in the response. Nonetheless, considering that the full length antigen was expressed, it is possible that the release of IFN γ seen is due not only to activation of CD8⁺T cells but also of CD4⁺T cells.

From the previous chapter, more cells seemed to be draining to the LN following virus rather than infected DC injection (figure 4.7), suggesting that the former method would result in a greater immune response. Interestingly, after a single immunisation, injection of infected DCs resulted in a stronger anti-NP response than virus injection. Because studies with HSV-1 have only looked at injection of virus and not of infected DCs, the results presented here need to be compared to findings obtained using different vector systems. Matsui et al (Matsui et al., 2002) and Wan et al (Wan et al., 1999) compared the two methods using adenoviruses expressing the HCV core gene or gp100 respectively. This showed that in both cases infected DCs were more efficient at stimulating an IFN γ responses in accordance with our HSV-1 results. On the other hand, third generation lentiviruses result in a more efficient induction of an IFN γ response after neat virus injection as compared to lentivirus infected DCs (Esslinger et al., 2003). It appears, therefore, that the type of virus determines the most efficient method of

immunisation. In this study HSV-1 appeared to behave similarly to adenoviruses. Interestingly, whereas in adenovirus studies, administration of two immunising doses of either cells or virus did not change the advantage of injecting infected DCs over virus (Brossart et al., 1997), with HSV1, a second dose, according to the ELISPOT data, abrogated the response induced by the infected cells, whereas that from virus injection remained unchanged. In a boosting regime, immunisation with neat virus therefore seems to be more advantageous.

The absence of an immune response to NP following a second injection of infected DCs and the lack of improvement in IFN γ stimulation when using neat virus is paralleled and may be due to the strong antiviral response after primary and secondary immunisation. This has already been detected in other studies using various vectors such as *Toxoplasma gondii*, in which case even primary responses to *Plasmodium yoelii* circumsporozoite protein (PyCS) were prevented by strong anti-vector immunity (Charest et al., 2000). Similarly, repetitive injection of vaccinia or influenza virus expressing the PyCS protein have been shown not to increase the number of activated cells compared to single immunisation (Murata et al., 1996). To avoid this, the secondary responses should be induced by different priming and boosting vectors, which often should be administered in a specific order (Murata et al., 1996).

A response to the virus following the first DCV or V injection is expected, footpad injection of HSV1 stimulates an anti-viral IFN γ dependent response, primed by CD11c⁺CD8 α ⁺CD45RA⁻dendritic cells (Smith et al., 2003), very shortly after injection (Mueller et al., 2003), and is targeted mainly to the gB antigen (Mueller et al., 2003, Wallace et al., 1999). This IFN γ response, on one hand, has been shown to be an essential factor for clearance of the HSV1 from cutaneous sites of infections, and a single footpad immunisation with attenuated HSV1 has previously resulted in a response strong enough to be protective against subsequent viral challenges (Brehm et al., 1999). On the other hand, other studies have shown that IFN γ is not required for virus clearance (Milligan & Bernstein, 1997) or for protection (Bouley et al., 1995, Ghiasi et al., 2000). These controversial studies imply that the antiviral response induced from the first

inoculation may be strong enough to fully clear in some cases, or partially in others, the incoming virus from the second injection. To clarify this point, Brockman and Knipe (Brockman & Knipe, 2002) have investigated the effect of previous immunisations to HSV1 on the efficiency of an incoming recombinant HSV1 to stimulate an IFN γ and antibody responses both to the virus and to the recombinant antigen. No adverse effect was reported in this case from previous immunisation and, similarly, when using an HSV amplicon encoding HIVgp120, previous immunisation resulted in only a slight reduction of response (Hocknell et al., 2002).

Taken together, these reports imply that although a strong antiviral response is induced by footpad injection of virus, this should not, or only minimally, influence subsequent administrations of virus. The results presented here are in agreement with this model since, as in the experiments referred to, despite the strong antiviral response, administration of two doses of neat virus did not alter the anti-NP response (which was not increased either). Nonetheless, the anti-HSV1 response induced by infected DCs has not previously been investigated but here has resulted in a stronger immune response when using virus alone. Probably there is a certain threshold above which there is complete clearance of the subsequent incoming infected cells and abrogation of IFN γ response to NP. This model is supported by the fact that immunisation with peptide pulsed DCs, to which no other response is mounted from the primary infection, gives a similar response independent of boosting

Overall, although it is possible to stimulate an anti-NP response by DCV and virus injection, a second injection did not confer any advantage to virus injection but rather inhibited the response from infected cells. Moreover, although following a single immunisation infected DCs induced a stronger anti-NP response than virus injection, from a vaccine prospective, immunising with neat virus is more reliable at ensuring a response in case of previous exposure to the virus.

well as in studies using vaccinia virus expressing HIV Env (Rodriguez et al., 1999). Additional injection of recombinant adenovirus expressing GMCSF upon BCG vaccination (Wang et al., 2002) or in conjunction with live attenuated vaccines such as clamydia (Lu et al., 2002) have shown an increase and prolongation of the immune response in the first case, and even to induce protection in the second case when compared to vaccination without GMCSF. Similarly, co-injection of a plasmid expressing mGMCSF with one expressing the CS protein of *P. yoelii*, significantly improved protection (Weiss et al., 1998). Incorporation of GMCSF directly as part of the live attenuated parainfluenza 3 vaccine has also been shown to be beneficial in non human primate (Bukreyev et al., 2002).

All these studies showed an increase in CD4, CD8, or $\text{INF}\gamma$ responses, together with antibody production, showing that GMCSF has an effect on the stimulation of both cellular and antibody responses. Interestingly, although transient expression of GMCSF can potentially promote CD8 and CD4 T cell responses and increase the number of $\text{INF}\gamma$ releasing cells, the level of cytotoxic CTL response is not usually affected (Weiss et al., 1998). In addition, GMCSF has been found not to change the type of response usually stimulated, but rather to amplify ongoing responses (Morrissey et al., 1987, Wang et al., 2000).

Surprisingly, no significant difference could be seen in the experiments reported here by the addition of GMCSF when injecting neat virus. However, on the other hand, co-expression of GMCSF in infected cells induced a response stronger than was seen following injection of cells infected with the virus not expressing GMCSF.

The fact that a stronger response is obtained from DCV than from virus even in experiments where no GMCSF is involved, implies that ex-vivo infected DCs produce and present a larger amount of protein (both NP and GMCSF). The fact that no improvement is seen in neat virus injection by the addition of GMCSF may therefore be due to the insufficient amount of GMCSF produced and released. The amount of GMCSF needed to stimulate an increase in the response

is not very high, a serum level of 8-10ng/ml is enough to have a beneficial effect (Miller et al., 2002). In fact, overexpression of GMCSF in vivo (55ng/ml) by an adenovirus vector has actually resulted in fatal toxicity in nearly 50% of mice within two weeks (Miller et al., 2002). In addition, studies by Peretz (Peretz et al., 2002) and Basak (Basak et al., 2002) have shown that repeated or continuous injections of GMCSF do not further increase the number of DCs found in the spleen than are found following the first injection and this reaches a plateau following administration of 10µg/day for 7 days.

In the experiments presented here, GMCSF could not be detected in the supernatant of 10^6 infected DCs, but despite this, infected DCs might still produce sufficient GMCSF to have a beneficial effect. On the other hand, virus injection does not target as many cells as ex-vivo infection, and therefore, although the amount of antigen delivered and presented is enough to stimulate a response, the amount of GMCSF produced may not be high enough to allow it to have an effect.

Taken together these results show that ex-vivo infected DCs present and express antigen more efficiently than neat virus injection. This has the adverse effect of inducing a very strong antiviral response which does not allow boosting, but contrarily to virus injection, has the advantage of producing enough GMCSF to enhance the response following a single immunisation. When using viral vectors for immunotherapy, a balance between the response to the virus and to the recombinant protein is essential: injection of neat virus seems to achieve this goal since previous exposure to the virus does not affect the efficiency of the incoming vector. In addition, co-expression of GMCSF is recommended for virus injection even if it does not enhance the response, since this appears to increase the reproducibility of the results. An improvement in the anti-NP response after immunisation with the GMCSF expressing virus could probably be achieved by additional expression of a DC-targeting molecule such as CD40 ligand.

Chapter 6

HSV-1 vectors applied to malaria immunotherapy

6.1 Introduction

The 17⁺47NP/vhsVP16⁻ virus has been demonstrated to be able to stimulate IFN γ responses to the test antigen NP. It was therefore decided to investigate the ability of this backbone to induce an immune response to malaria antigens. Malaria infection comprises both intracellular and extracellular stages. Upon biting, the anopheles injects *sporozoites* that rapidly migrate to the liver and infect the hepatocytes. Within the hepatocytes, the sporozoites replicate producing *merozoites* that are released from the liver cells and in turn infect and replicate in red blood cells: the successive cycles of infection of red blood cells are responsible for the onset of the symptoms of malaria. Targeting the pre-erythrocytic stage of the disease would block the initial infection of hepatocytes and successive formation of merozoites. Mice (Weiss et al., 1988) and humans (Malik et al., 1991) immunised with irradiated sporozoites are in fact able to inhibit parasite development in the liver (Khusmith et al., 1991), and show strong CD8⁺T and CD4⁺T cell response mainly against the circumsporozoite protein (CS) and sporozoite surface protein 2 (SSP2), also known as thrombospondin-related anonymous protein (TRAP), thus making CS and SSP2, two main candidates for inclusion in malaria vaccines.

Of the numerous strains of malaria, *Plasmodium yoelii*, one of the strains of murine malaria, has been extensively studied, where protective immune mechanisms against the pre-erythrocytic stages have been well characterised (Nardin & Nussenzweig, 1993). Genes from *plasmodium yoelii* were therefore used for the work in this chapter. CS is the most abundant protein on the surface of the sporozoites, and allows the sporozoites to bind to hepatocytes. CS is also involved in the formation of sporozoites in the plasmodium oocysts, and in the binding of sporozoites both to the plasmodium salivary glands, and to ribosomes in the host, thus inhibiting protein synthesis. Taking into account these functions, inducing an immune response against CS may result in significant protection against sporozoite invasion.

CS is a transmembrane protein with a conserved structure throughout the different strains of plasmodium. PyCS from *plasmodium yoelii* (PyCS) is 367 amino acids

in length, with a molecular weight of ~60kDa with an amino terminal signal sequence and an anchor sequence at the carboxy terminal. Following the signal sequence, a stretch of aminoacids (pre-region I repeats) precede region I, which, together with region II, are conserved throughout the plasmodium species. The repeat domain of the protein is comprised between region I and region II and is slightly unusual in the *p. yoelii* protein, having two groups of repeats, the first set of 6 amino acids and repeated 15 times and the second set composed of 4 amino acids repeated 8 times (Lal et al., 1987). Finally, between the tetramer repeats and region II residues is the CD8⁺CTL immunodominant domain SYVPSAEQI (aa280-288), the main target of cellular immune responses.

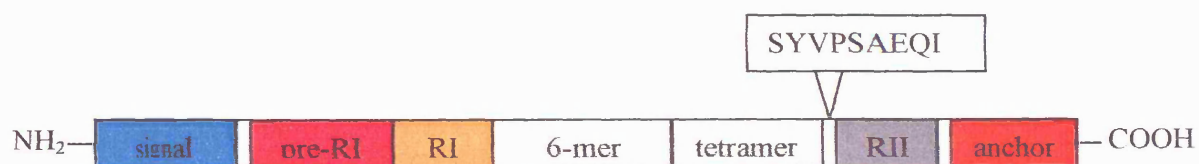


Figure 6.1: Schematic diagram of PyCS

Immunisation with a recombinant adenovirus expressing the full length PyCS (AdPyCS) has been shown to induce CD8⁺CTL cells as well as CD4⁺T cell and antibody responses, and to be sufficient to confer protection against challenge, (Rodrigues et al., 1997).

The second immunodominant protein of the pre-erythrocytic stage is sporozoites surface protein 2 (SSP2). Studies have shown SSP2 to be essential for the gliding motion of the sporozoite and for cell invasion (Menard, 2000), making the SSP2 gene an equally promising target for vaccines. PySSP2 is 826 amino acids in length, 140kDa protein, with the immunodominant CD8⁺T cell peptide (KYIFVLLLL) between amino acid 8-16 (Hettihewa, 2003). Similarly to PyCS, PySSP2 contains a middle sequence of 6 amino acids tandemly repeated and flanked by non-repetitive sequences (Rogers et al., 1992).

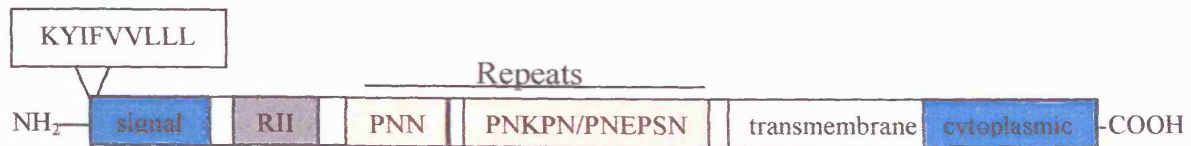


Figure 6.2: Schematic diagram of PySSP2

Individuals who are naturally exposed to *plasmodium falciparum* have been shown to generate immune responses to SSP2 (Flanagan et al., 2003), and immunisation with cells expressing SSP2 resulted in CD8⁺T cell dependent protection in 33-67% of immunised mice upon sporozoite challenge (Khusmith et al., 1991). Similarly, passive transfer of CD8⁺ T cell clones raised against PySSP2 could induce immunity against sporozoite challenge (Khusmith et al., 1994) by eliminating infected hepatocytes (Khusmith et al., 1991, Khusmith et al., 1994), and injection of irradiated sporozoites could induce both CTL (Khusmith et al., 1991) and antibody responses (Charoenvit et al., 1987) in mice. These observations make CTL responses to SSP2 a promising target of pre-erythrocytic vaccines, and suggest that the use of full length genes would be appropriate since both CD4⁺ and antibody responses would be expected to be induced too.

Considering the immunogenicity of both CS and SSP2, and the ability of the 17⁺47⁻vhs⁻VP16⁻ vector to induce CD8⁺CTL responses, the work in this chapter aimed at using this viral backbone to express the PyCS or PySSP2 genes and to stimulate a CD8⁺CTL response to both proteins. In addition, the possible advantage of coexpressing GMCSF from the virus suggested by the previous chapter will be further investigated.

Viruses used in this chapter

This diagram indicates the progression of virus construction
Specific deletions and inactivations of each backbone are specified

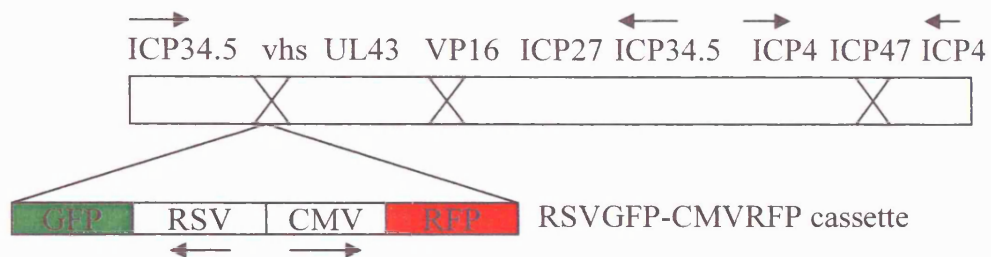
X indicates inactivity of the gene either by deletion or inactivation
→ indicates the direction of gene expression

17⁺47⁻ RSVGFP-CMVRFP/vhsVP16⁻

ICP47 gene deleted

vhs gene inactivated by insertion of the RSVGFP-CMVRFP cassette

VP16 gene with V422 mutation

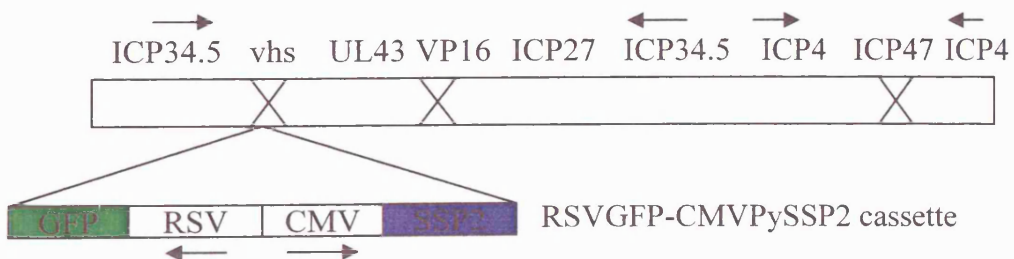


17⁺47⁻ RSVGFP-CMVSSP2/vhsVP16⁻

ICP47 gene deleted

vhs gene inactivated by insertion of the RSVGFP-CMVPySSP2 cassette

VP16 gene with V422 mutation

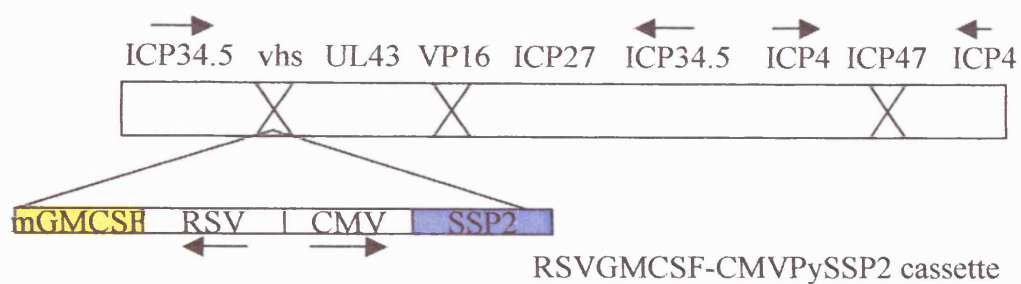


17⁺47⁻ RSVGMCsf-CMVSSP2/vhsVP16⁻

ICP47 gene deleted

vhs gene inactivated by insertion of the RSVGMCsf-CMVPySSP2 cassette

VP16 gene with V422 mutation

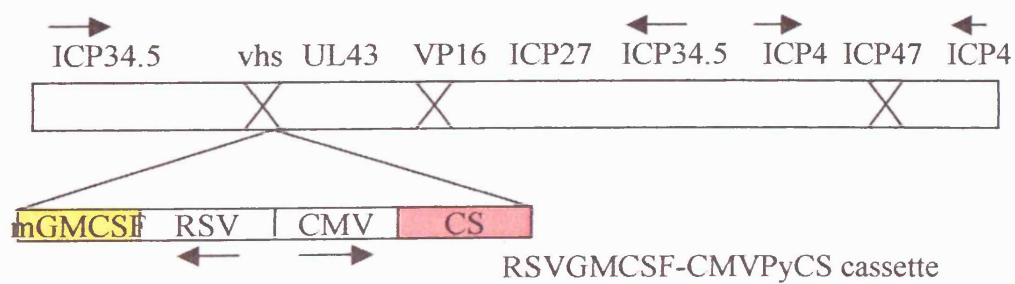


17⁺47⁻ RSVGMCsf-CMVCS/vhsVP16⁻

ICP47 gene deleted

vhs gene inactivated by insertion of the RSVGMCsf-CMVPyCS cassette

VP16 gene with V422 mutation



6.2 Use of 17⁺vhs⁻47⁻VP16⁻ backbone to stimulate an immune response to Plasmodium yoelii sporozoite surface protein 2 (PySSP2)

6.2.1 Construction of 17⁺47⁻GFP-SSP2/vhs VP16⁻

The level of expression of recombinant genes from cassettes inserted in the HSV1 genome has been shown to depend on the promoters used and on whether a double or single promoter expression cassette is used (Palmer et al., 2000). The work presented so far in this thesis has mainly used cassettes expressing a single gene, except in the construction of 17⁺47⁻GMCSF-NP/vhsVP16⁻. The effect of expressing GMCSF from the virus has therefore been studied comparing two viruses differing in the type of cassette inserted. In this chapter, two identical viruses containing double expressing cassettes one of which expresses GFP and one mouse GMCSF (mGMCSF) in addition to the recombinant PySSP2 were constructed and compared for their ability to induce immune responses.

The PySSP2 gene was amplified by PCR from the genome of 17XNL *Plasmodium yoelii* using primers containing a BamHI site (§ 2.2.11). The 2.5Kb gene was cloned into the pGemTEasy vector forming pGemTeasyPySSP2, and sequenced. To construct a plasmid expressing both GFP and SSP2, hrGFP was purified from p_{hr}GFP by digestion with NotI and XhoI, it was then blunted, and inserted into p_{vhs}DC at the PmeI site following the RSV promoter, thus forming p_{vhs}DCGFP. To insert PySSP2 into the p_{vhs}DCGFP plasmid, the PySSP2 gene was purified from pGemTEasyPySSP2 using NotI, and was blunted before cloning into the blunted ApaI site of p_{vhs}DCGFP to obtain p_{vhs}DCGFP-SSP2 in which hrGFP is driven by the RSV promoter and PySSP2 by the CMV promoter (figure 6.3).

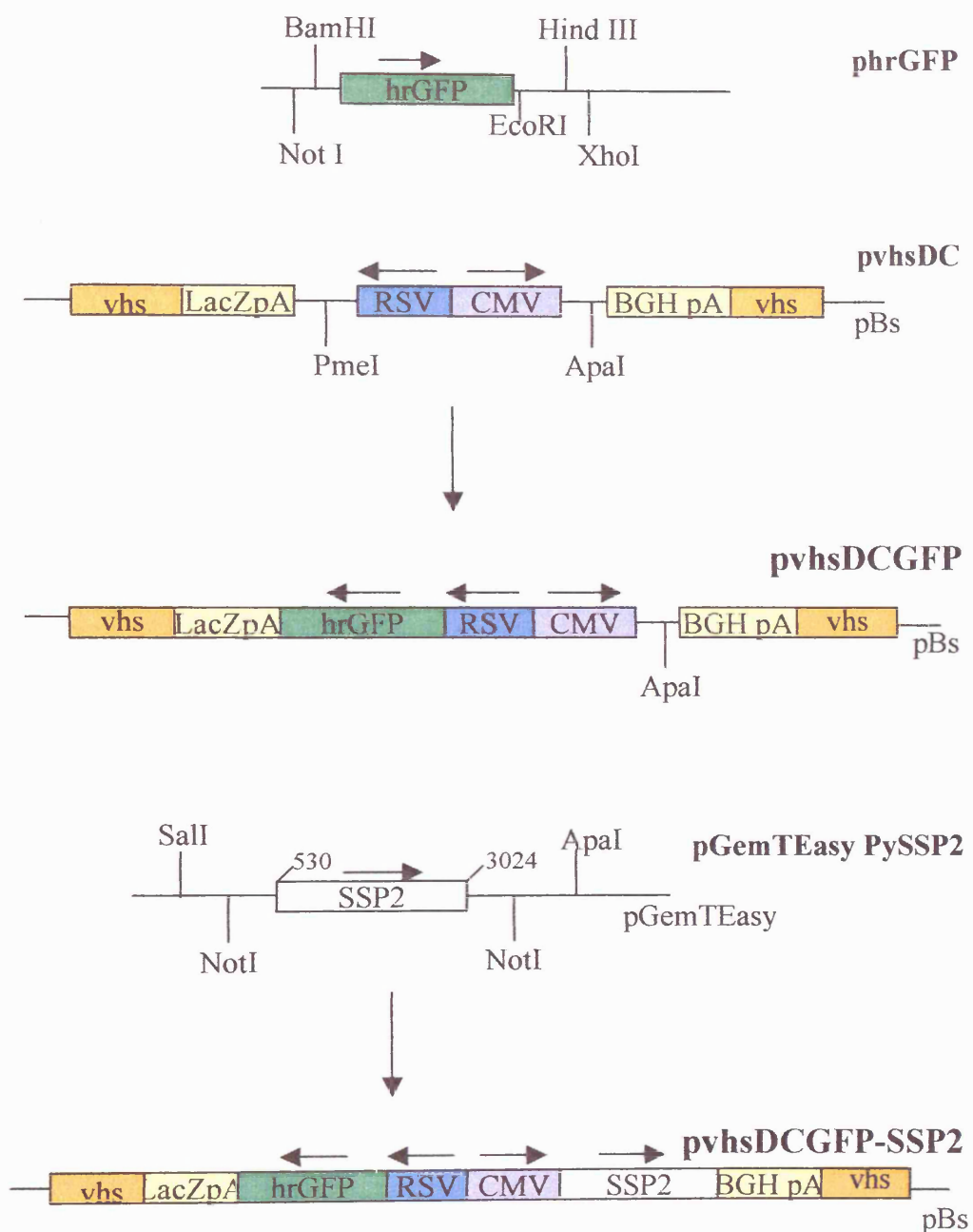


Figure 6.3: Construction of pvhsDCGFP-SSP2 plasmid
Cloning strategy for the construction of the plasmid encoding both the GFP and PySSP2 genes.

The pvhsDCGFP-SSP2 plasmid was transfected into BHK cells together with 17⁺vhs⁻47⁻VP16⁻ viral DNA and recombinant green fluorescent plaques purified. Southern blotting was used (figure 6.4a) to confirm the presence of the PySSP2 gene. Here, viral DNA was digested with BamH1 with sites at either sides of the inserted 2.5Kb PySSP2 gene which was identified using a 560bp probe internal to SSP2 (bp760-bp1320 obtained by SspI digestion of pGemTEasyPySSP2) (figure 6.4a lane7). Expression of PySSP2 from six purified plaques was subsequently checked by running a 5% SDS PAGE gel (figure 6.4b) followed by western blotting which showed strong expression from all plaques, with a band appearing at ~160kDa, corresponding to PySSP2, the theoretical molecular weight of which is ~140kDa. This band was not present in the sample of cells infected with 17⁺47⁻ RFP-GFP/vhsVP16⁻. Plaque 1 was used for further experiments.

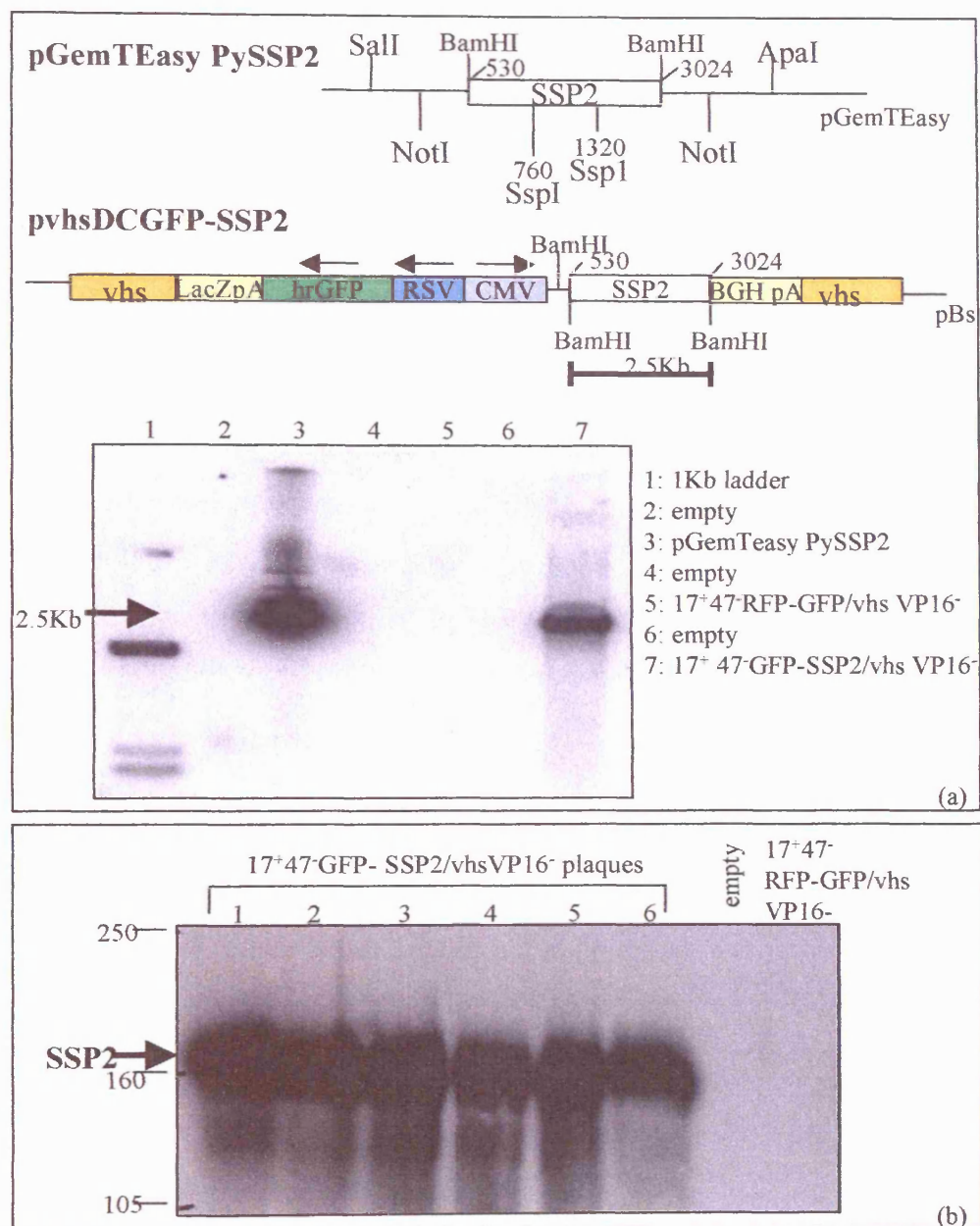


Figure 6.4: Detection of recombinant SSP2

(a) Southern blot of 17⁺47-GFP-SSP2/vhsVP16⁻. Viral DNAs and plasmid control (pGemTEasyPySSP2) were digested with BamHI and probed with 560bp fragment from pGemTEasyPySSP2 cut with SspI.

(b) Western blot of lysates of BHK cells infected with 17⁺47-GFP-SSP2/vhsVP16⁻ plaques (1-6), or with 17⁺47-RFP-GFP/vhsVP16⁻ (-ve control) using a polyclonal anti-SSP2 antibody (§2.1.5, 2.2.16).

6.2.2 Construction of 17⁺47⁻GMCSF-SSP2/vhsVP16⁻

To construct a plasmid containing both the mGMCSF and PySSP2 genes driven by the RSV and CMV promoters respectively, PySSP2 was excised from pGemTEasyPySSP2 with NotI, blunted and cloned into the Pml I site of pvhsDCGMCSF plasmid (§ 5.3.2, figure 5.11), to form pvhsDCGMCSF-SPP2 (figure 6.5).

The pvhsDCGMCSF SPP2 plasmid was transfected into BHK cells together with 17⁺47⁻RFP-GFP/vhs VP16⁻ viral DNA. Plaques that were neither green nor red fluorescent were purified, and two pure plaques characterised by Southern blot for the presence of the PySSP2 gene. Viral DNAs, including that from 17⁺47⁻RFP-GFP/vhsVP16⁻ as a negative control, were digested with BamHI, and the blot probed with a 630bp fragment released by SspI digestion of pGemTEasyPySSP2 (figure 6.6a, lanes 6,7). Expression of PySSP2 was confirmed by western blot where a ~160kDa protein was detected (figure 6.6b lanes 1,2).

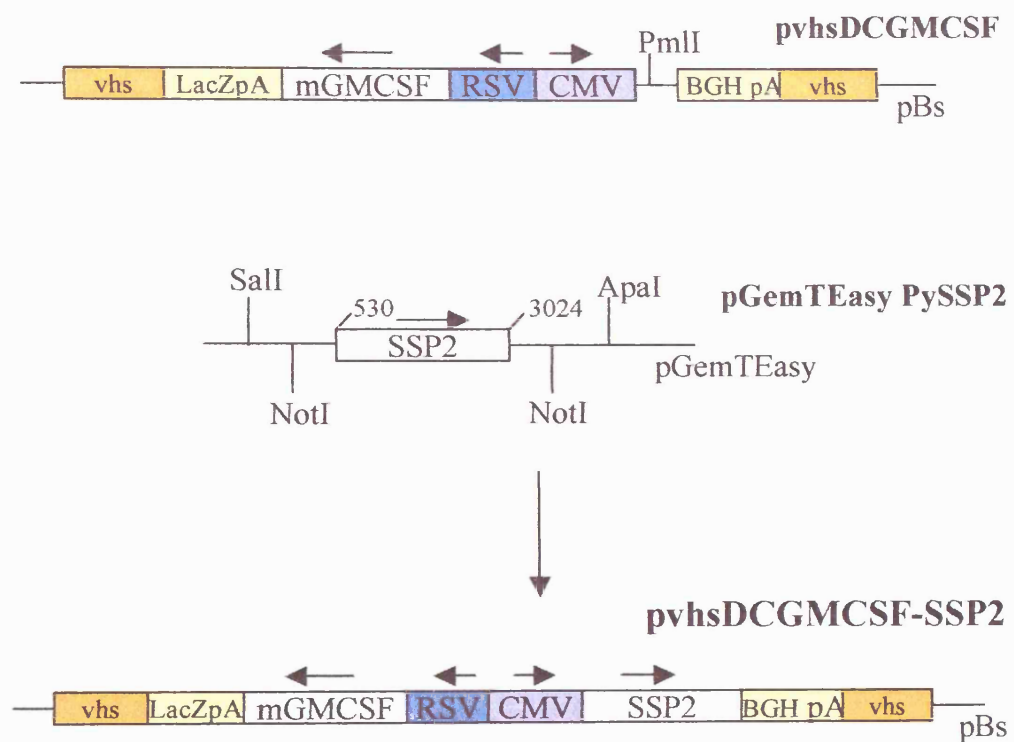


Figure 6.5: Construction of pvhsDCGMCSF-SSP2

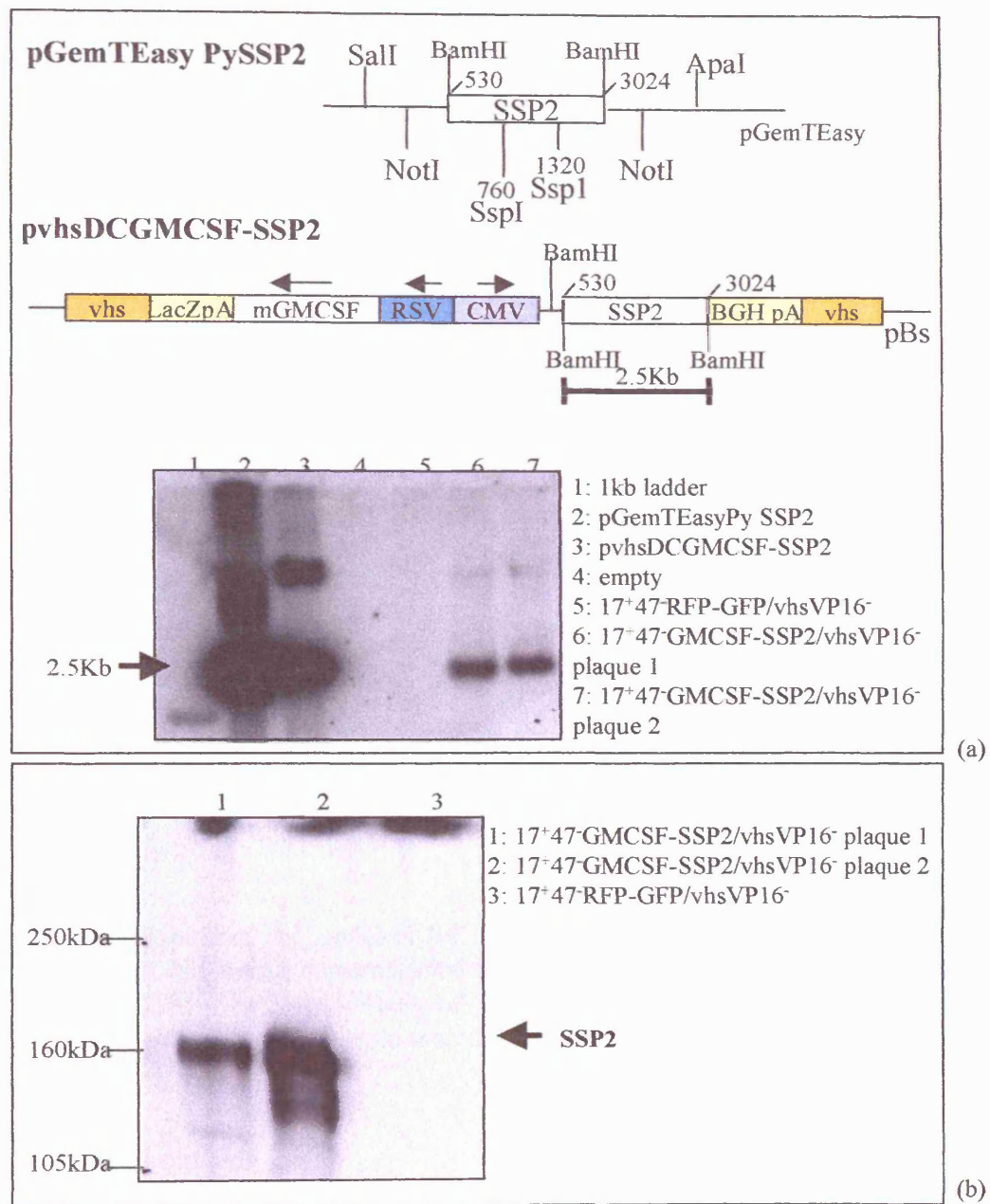


Figure 6.6: Detection of recombinant PySSP2

(a) Southern blotting of 17⁺47-GMCSF-SSP2/vhsVP16⁻ DNA (lane6-7). Viral DNAs and plasmids were digested with BamHI and probed with 560bp fragment from pGemTeasySSP2 digested with SspI.

(b)Western blotting using lysates of BHK cells infected with two different plaques of 17⁺47-GMCSF-SSP2/vhsVP16⁻ (lane 1-2) or with 17⁺47-RFP-GFP/vhsVP16⁻ as negative control (lane 3) probed with an anti-SSP2 antibody (§materials and methods).

Expression of mGMCSF gene by $17^{+}47$ GMCSF-SSP/vhsVP16⁻ was tested by ELISA on the supernatant of BHK cells infected with either this virus or, as negative control, with the $17^{+}47$ GFP-RFP/vhsVP16⁻ virus (figure 6.7). This confirmed the expression of GMCSF from the appropriate virus. Plaque 2 was grown to large stocks for future experiments.

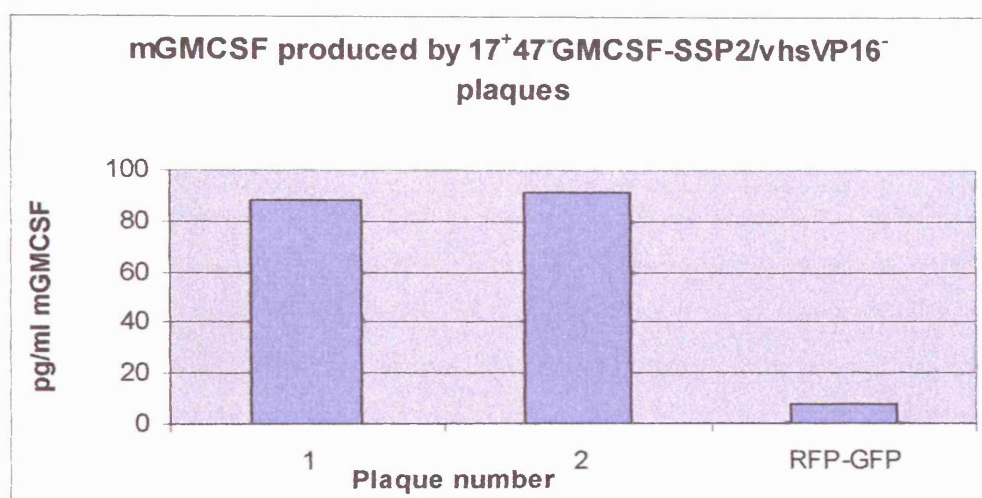


Figure 6.7: **Production of mGMCSF from $17^{+}47$ GMCSF-SSP2/vhsVP16⁻** ELISA for mGMCSF using supernatants from BHK cells infected for 24hr with either $17^{+}47$ GMCSF-SSP2/vhsVP16⁻, or $17^{+}47$ RFP-GFP/vhsVP16⁻. The values shown are the average of each sample tested in duplicate.

6.2.3 Expression of PySSP2 in infected dendritic cells

Although the ability of the HSV-1 17⁺vhs⁻47⁻VP16⁻ backbone to infect and express genes in dendritic cells had already been demonstrated for GFP and NP (§3.5, 5.2.), expression of mGMCSF and PySSP2 was tested in infected DCs using Western blot. It was possible to detect SSP2 expression after infection with either 17⁺47⁻GFP-SSP/vhsVP16⁻ or 17⁺47⁻GMCSF-SSP/vhsVP16⁻ and not in uninfected DC (figure 6.8a).

Expression of mGMCSF from DCs infected with 17⁺47⁻GMCSF-SSP2/vhsVP16⁻ was assessed by ELISA (figure 6.8b), and similar to the results from the virus expressing mGMCSF and NP (§5.3.3), only very low levels of mGMCSF could be detected in the supernatant of 10⁶ infected DCs.

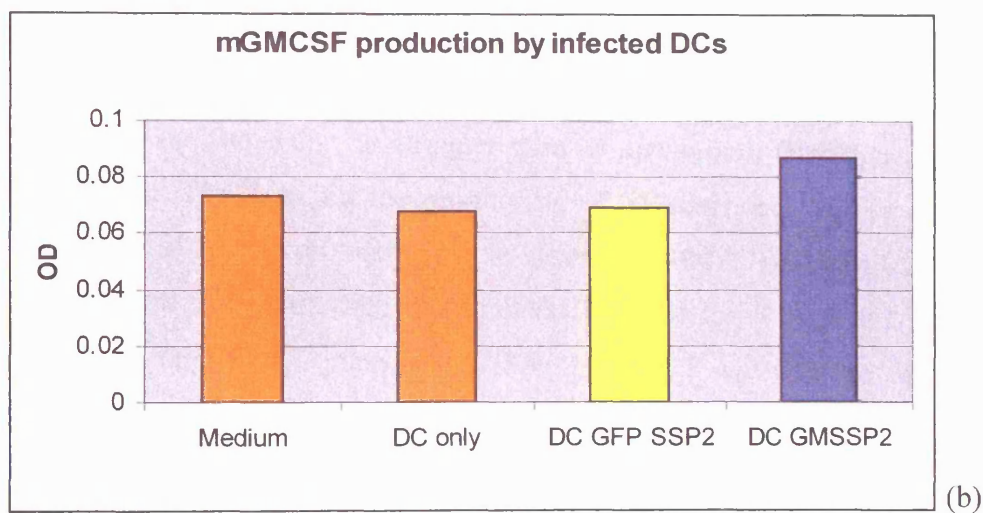
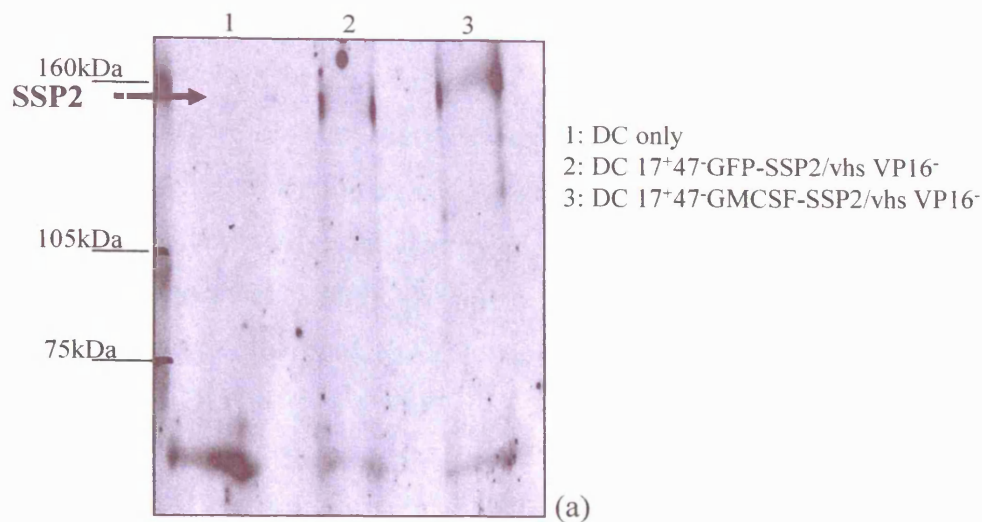


Figure 6.8: Expression of SSP2 and mGMCSF from infected DCs

(a) Anti-SSP2 western blot using 10^6 DC either non-infected (DC only), or infected with 17⁺47-GFP-SSP2/vhsVP16⁻, or 17⁺47-GMCSF-SSP2/vhsVP16⁻ at MOI=1.

(b) mGMCSF ELISA using supernatants of DCs uninfected (DC only), or infected with 17⁺47-GFP-SSP2/vhsVP16⁻ (DCGFPSSP2), or 17⁺47-GMCSF-SSP2/vhsVP16⁻ (DCGMSSP2) at MOI=1. OD values are presented rather than quantitative values since these were lower than the standard curve minimum.

6.2.3 Ability of viruses containing the full length PySSP2 gene to induce an IFN γ immune response and the effect of mGMCSF on the response

Despite the fact that previous experiments with ex-vivo manipulated DCs had resulted in high levels of background in the ELISPOT assay (§5.2.2, 5.2.3), it was decided to investigate both ex-vivo infected DCs and in vivo targeted DCs as possible routes of immunisation especially taking into account that DCs have been suggested to be functionally inhibited by plasmodium infection (Ocana-Morgner et al., 2003, Urban et al., 1999). Groups of three mice were immunised in the flank and footpad with 10^6 mature DCs loaded with the CD8⁺CTL SSP2 peptide (KYIFVVLLL) (DCSSP), 10^6 DCs infected with the GFP-SSP2 virus (DCVGFPSSP) or with the GMCSF-SSP2 virus (DCVGMSSP), or 10^6 pfu of either viruses alone (VGFP and VGMSSP). Non-immunised mice and mice that received 10^6 mature DCs (DC only) were used as negative control. Considering that studies looking at malaria responses have shown that the response obtained two weeks post immunisation is stronger than at one month (Rodrigues et al., 1997, Tsuji et al., 1998), as for the monitoring of NP responses, a 2 week time point was used for this experiment. Spleens were removed 13-14 days following immunisation, and the cells were restimulated in vitro for 7 days in medium containing the KYIFVVLLL peptide. An IFN γ ELISPOT was then set up using either peptide loaded A20 cells or naïve A20 cells as stimulators. The average number of spots obtained from the incubation with naïve A20 cells was subtracted from the average response obtained from incubation with peptide presenting cells, and the values obtained for each mouse and per group are presented in figure 6.9. Unfortunately, one sample from the DCVGMSSP group was lost during manipulation.

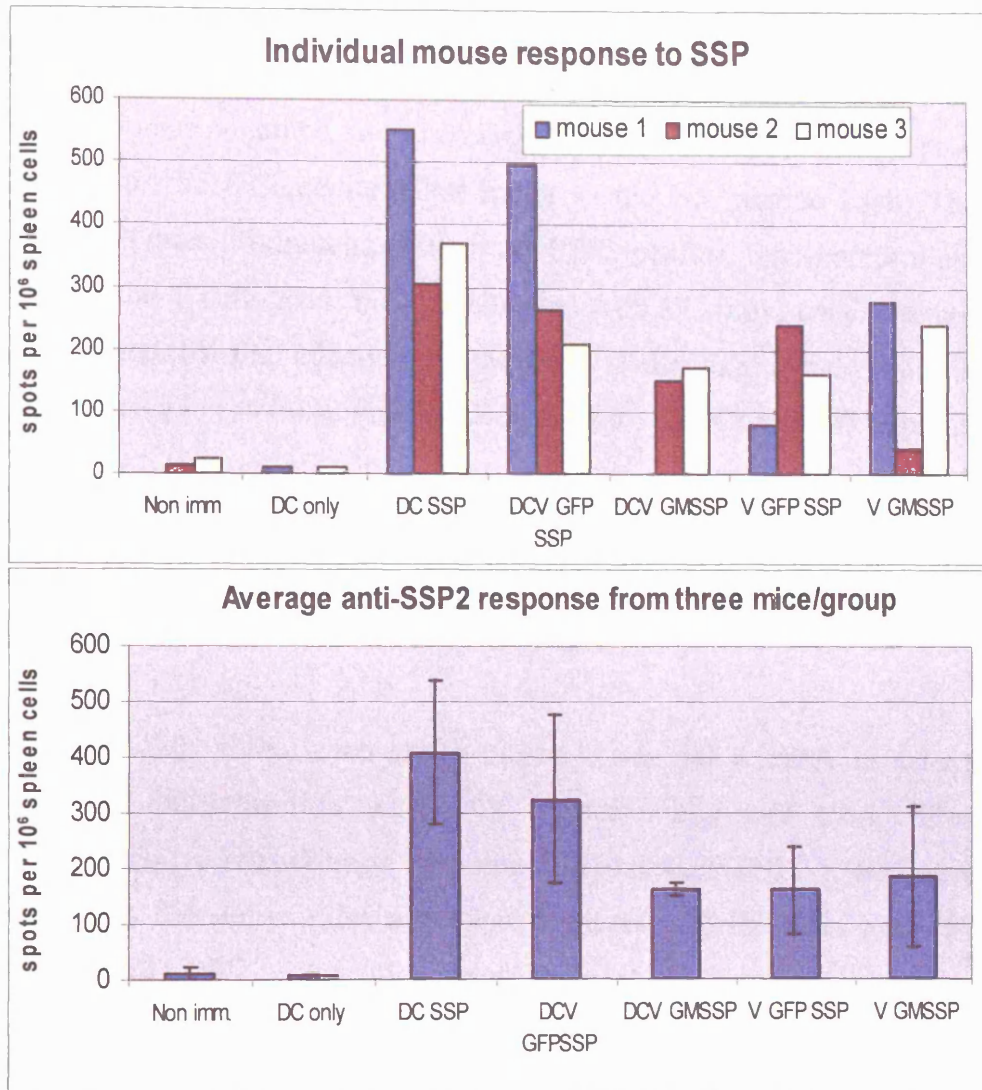


Figure 6.9: Anti-SSP2 IFN γ ELISPOT and effect of GMCSF expression

IFN γ response to SSP2 as measured by IFN γ ELISPOT assessing the effect of co-expressing GMCSF. Mice were immunised 2 weeks in footpad and flank with: nothing (non immunised), 10^6 LPS matured DC (DC only), 10^6 mature DC pulsed with SSP2 peptide (DCSSP), 10^6 DC infected at MOI of 1 with $17^{+}47$ -GFP-SSP2/vhsVP16 $^{-}$ (DCVGFPSSP), or infected at MOI 1 with $17^{+}47$ -GMCSF-SSP2/vhsVP16 $^{-}$ (DCVGMSSP), direct injection of 10^6 pfu of $17^{+}47$ -GFP-SSP2/vhsVP16 $^{-}$ virus (VGFPSSP), or direct injection of 10^6 pfu of $17^{+}47$ -GMCSF-SSP2/vhsVP16 $^{-}$ (VGMSSP). The values shown are for each mouse (a) or the average of three mice per group (b), the error bars represent the standard deviation.

The first encouraging result was the convincing SSP2 specific IFN γ response obtained from immunised mice. In this case, unlike with NP, no non specific response was seen in mice immunised with DC only (figure 6.9) or with a virus not expressing SSP2 (figure 6.10). This observation suggests that the non specific response previously obtained from incubation with the NP peptide was probably not only due to the DC medium, but rather to the NP peptide itself. The NP peptide may be more immunogenic than the SSP2 peptide, and therefore may be able to prime the T cells from mice immunised with DC only, which are already slightly activated by the injection of the cells. Following this hypothesis, no priming is observed in non-immunised mice in either the NP or SSP2 experiments as these T cells were not initially activated by an injection and the NP or SSP2 peptides therefore do not prime these naïve cells. The stronger efficacy of the NP peptide would also explain why the response seen in experiments using NP is around 10x higher than the one observed for SSP2.

The large variability within each group meant it was not possible to determine clearly whether immunisation with DCs or neat virus was more efficient. However, looking at the average responses to the GFP-SSP2 virus, ex-vivo infection of DCs seemed to raise a stronger response. On the other hand, when using the GMCSF-SSP2 virus, mice receiving neat virus appeared to respond slightly better.

In order to further define the effect of co-expressing GMCSF, groups receiving the same immunisation (DCs or virus) were compared. Surprisingly, and contrarily to that observed for NP (§5.3.4), co-expression of GMCSF from the ex-vivo infected DCs, did not result in an increased immune response, but rather a lower but more reproducible response was obtained. On the other hand, upon injection of neat viruses, expression of GMCSF resulted in the suggestion of a slight advantage provided by GMCSF.

Due to the high variability within groups, the suggestion that the addition of GMCSF conferred an advantage when neat virus was injected was investigated further using larger groups of 10 mice each. The larger sample group clearly

induce a stronger response than a virus only expressing SSP2 but rather, as for immunisation with infected DCs, GMCSF produces a decrease in the number of responding cells but gives more reproducible results, which is an important factor in vaccine development.

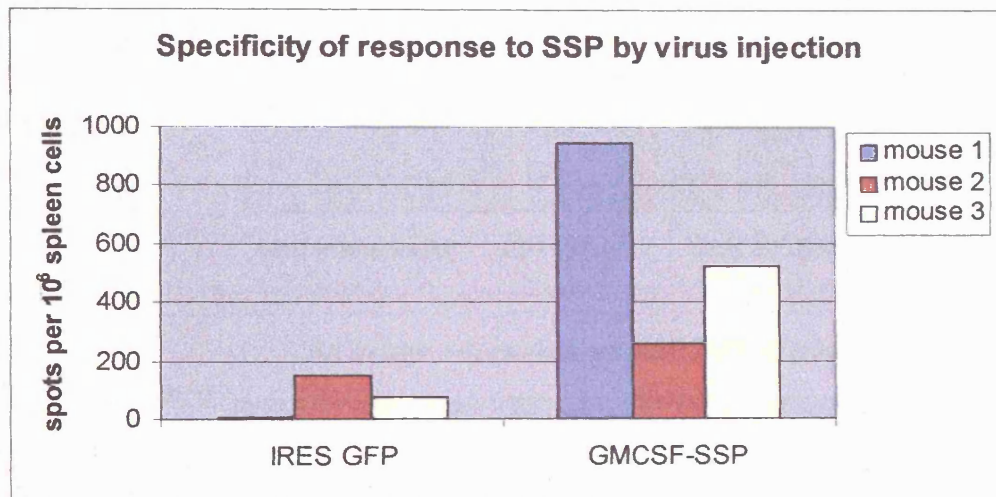


Figure 6.10: Specificity of response after virus injection

IFN γ ELISPOT measuring the anti-SSP2 response by individual mice after 2 weeks immunisation with 10^6 pfu of 17^+ vhs $^-$ 47IRESGFP/VP16 $^-$ (IRESGFP) or 17^+ 47GMCSF-SSP2/vhsVP16 $^-$ (GMCSF-SSP).

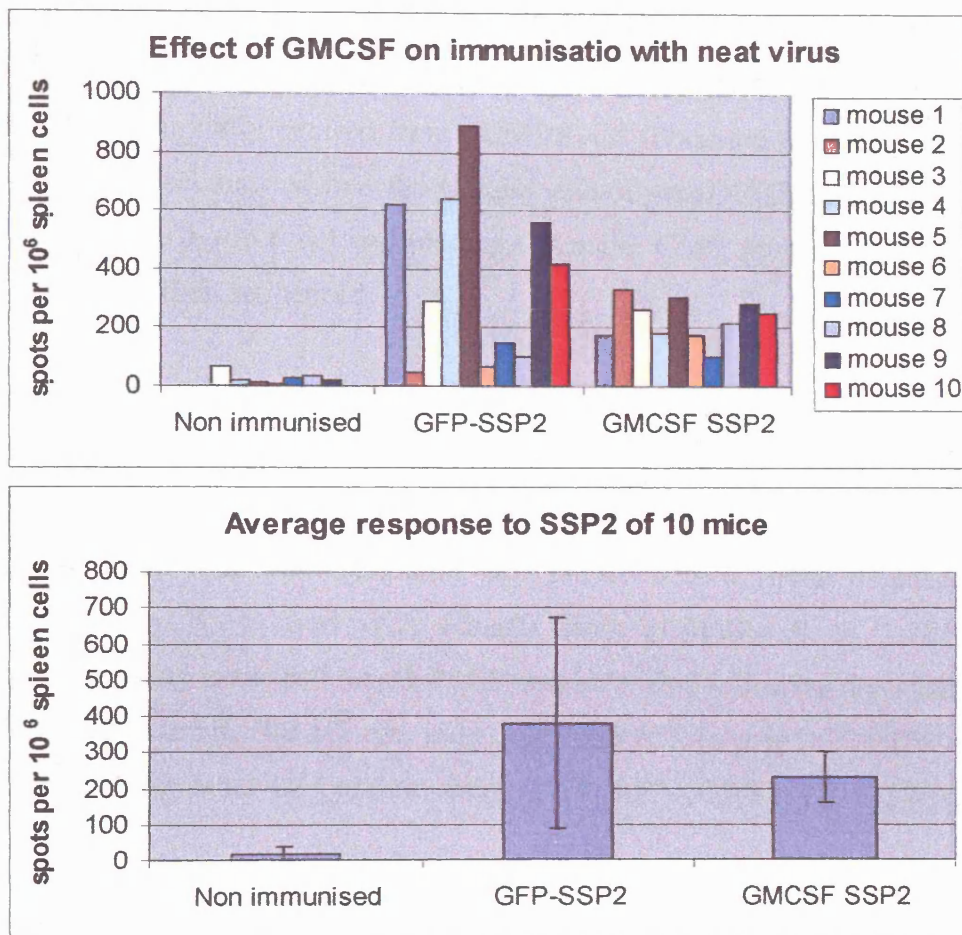


Figure 6.11: Effect of co-expression of GMCSF on anti-SSP2 immune response

Anti-SSP2 IFN γ ELISPOT using 10 mice per group following immunisation in the footpad and flank either non immunised, or immunised with 10^6 pfu of 17⁺47-GFP-SSP2/vhsVP16⁻ (GFP-SSP2), or 17⁺47-GMCSF-SSP2/vhsVP16⁻ (GMCSFSSP2). The immune response for each mouse is shown in (a) and the average response per group is shown in (b) with error bars representing the standard deviation.

6.3 Use of 17⁺vhs⁻47⁻VP16⁻ backbone to stimulate an immune response to *Plasmodium yoelii* circumsporozoite surface protein (PyCS)

6.3.1 Construction of 17⁺47⁻GMCSF-CS/vhsVP16⁻

For the construction of 17⁺47⁻GMCSF-CS/vhsVP16⁻ the nearly full length PyCS gene, missing the anchor sequence, was inserted into the pvhsDCGMCSF plasmid. PyCS was initially excised from pCMVPyCS (obtained from Dr Tsuji, NY) using BamH1 and inserted into the BamH1 site of pvhsDCGMCSF to form pvhsDCGMCSF-CS (figure 6.12) and placing CS under CMV promoter control. The inserted CS was then sequenced.

vhsDCGMCSF-CS was used for transfection into BHK cells with the 17⁺47⁻RFP-GFP/vhsVP16⁻ viral DNA to obtain recombinant, non-marker gene expressing plaques which were plaque purified, and six pure plaques were tested for presence of PyCS by Southern blot. The membrane was probed with a 764bp fragment (bp192-bp956 of PyCS) excised with HindIII from pCMVPyCS. A 1.1Kb fragment corresponding to the full length PyCS was identified following digestion of viral DNA with BamHI. BamHI cuts either side of PyCS in pvhsDCGMCSF-CS. (figure 6.13a). No band was present using 17⁺47⁻RFP-GFP/vhsVP16⁻ DNA (lane 3).

Expression of both PyCS and mGMCSF from the virus were confirmed by Western blot and ELISA respectively. Interestingly, two bands appeared on the Western blot around the expected size for PyCS (~60kDa) (figure 6.13b). Because the bands are so close, and at the right size, dimerisation or possibility of the protein being split into two have to be dismissed. Instead, the fact that malaria proteins are very unstable during refolding upon expression (Hetttihewa, 2003) and that problems in the purification of full length CS have previously been reported (Stratmann et al., 1997) suggest that the smaller band is possibly a degraded form of PyCS.

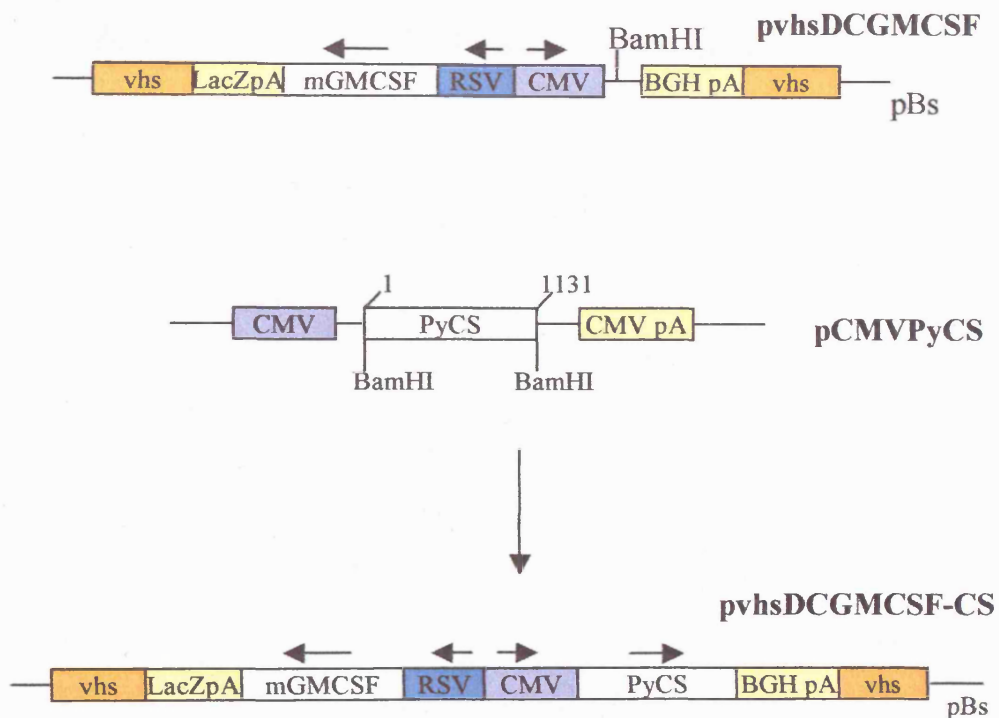


Figure 6.12: Construction of pvhsDCGMCSF-CS

Cloning strategy for the construction of the plasmid containing the double expression cassette between the vhs flanking regions and expressing mGMCSF from the RSV promoter and PyCS under the CMV promoter. PyCS was inserted at the BamHI locus of the plasmid already containing mGMCSF.

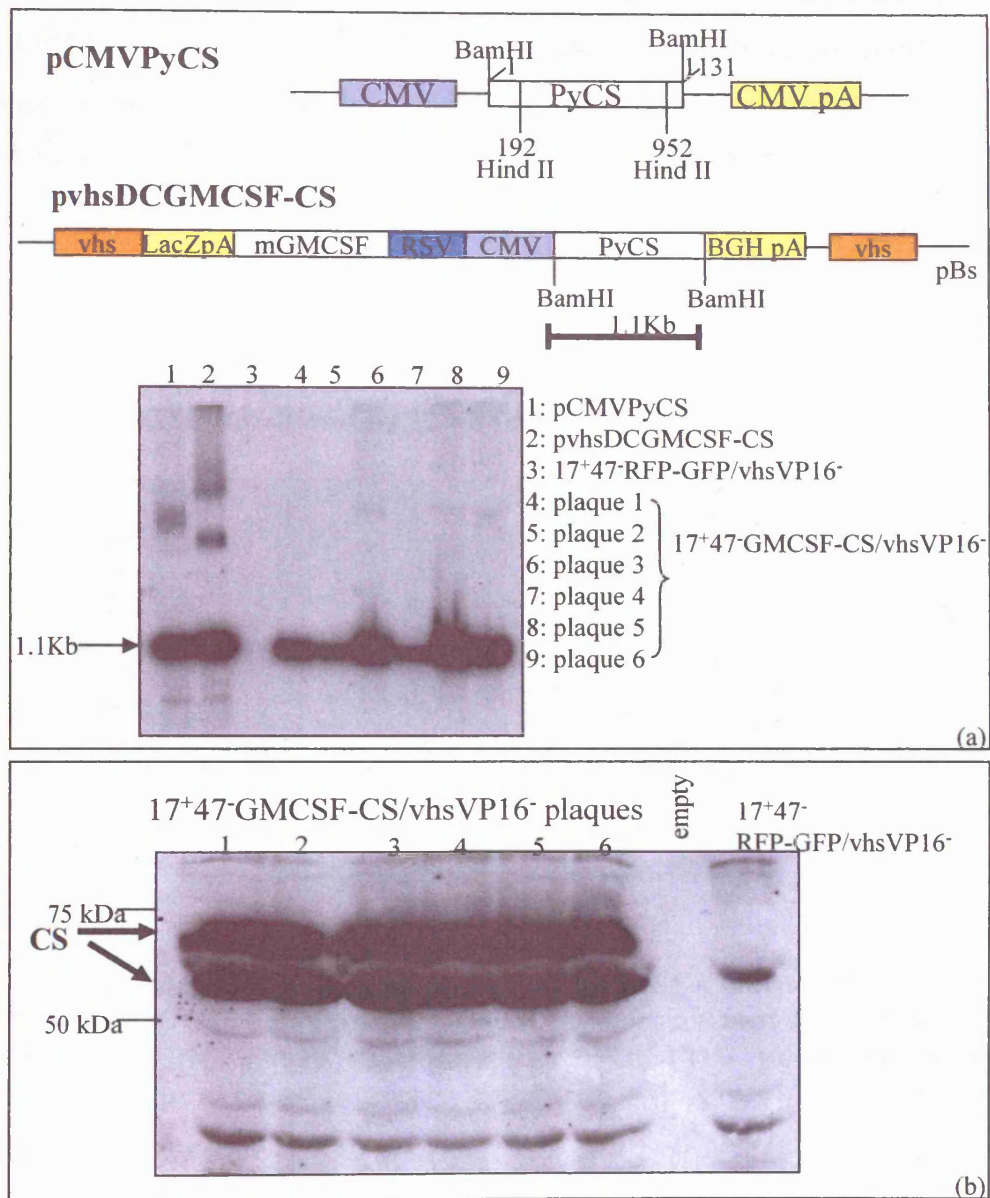


Figure 6.13:

Presence and expression of PyCS using 17⁺47-GMCSF-CS/vhsVP16⁻ infected BHKs

(a) Southern blot. Viral DNAs and plasmids were digested with BamHI and the membrane was probed with the 860bp CS fragment from Hind II digested pCMVPyCS.

(b) anti-CS (60kDa) Western blot using BHK cells infected with 17⁺47-GMCSF-CS/vhsVP16⁻ (1-6) or 17⁺47-RFP-GFP/vhsVP16⁻ (negative control).

All plaques of $17^{+}47\text{GMCSF-CS/vhsVP16}^{-}$ were also tested for their ability to express mGMCSF in non complementing cell lines (BHKs). The supernatants of infected BHKs were assayed for presence of mGMCSF and all the pure GMCSF-CS plaques were shown to produce mGMCSF, whereas the supernatant of BHK cells infected with $17^{+}47\text{RFP-GFP/vhsVP16}^{-}$ did not (figure 6.14).

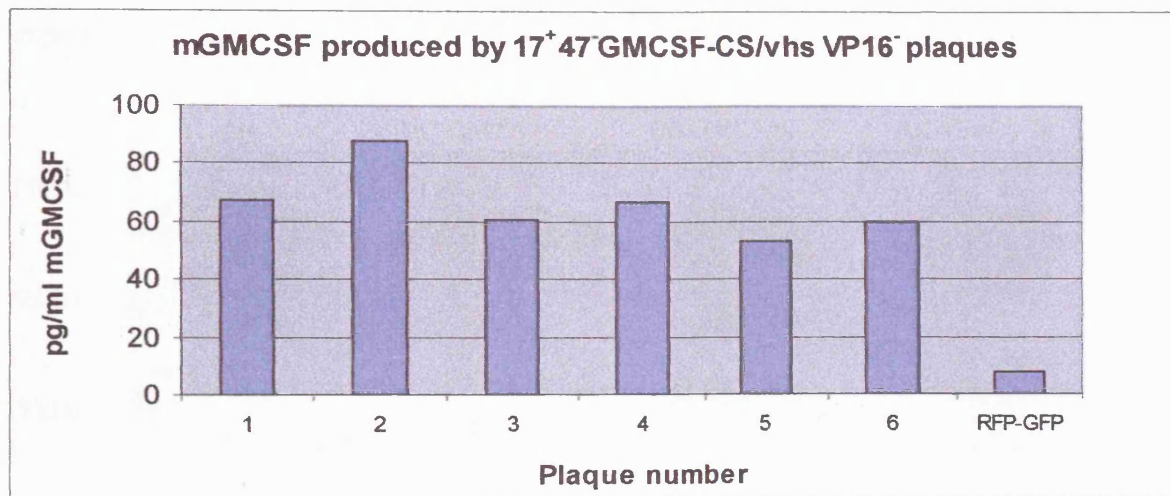


Figure 6.14: **mGMCSF expression from $17^{+}47\text{GMCSF-CS/vhsVP16}^{-}$**
mGMCSF ELISA using supernatant from BHK cells infected for 24hr with $17^{+}47\text{GMCSF-CS/vhsVP16}^{-}$ or $17^{+}47\text{RFP-GFP/vhsVP16}^{-}$. The values shown are the average of the value obtained from each sample run in duplicate

As before, expression in infected DCs was also tested. An SDS PAGE gel was run on lysates of 10^6 infected DCs and then western blotted to try to identify expression of PyCS. Unfortunately, a strong band corresponding to the size of PyCS also appeared in non-infected DCs (figure 6.15a), indicating cross reactivity of the antibody, thus making it impossible to specifically detect PyCS in DCs.

Production of mGMCSF from infected DCs was assessed using the supernatant from 10^6 DCs infected at various MOI (figure 6.15b). mGMCSF levels were very low, as before, and it was not possible to definitely concluded that mGMCSF was expressed in the DCs.

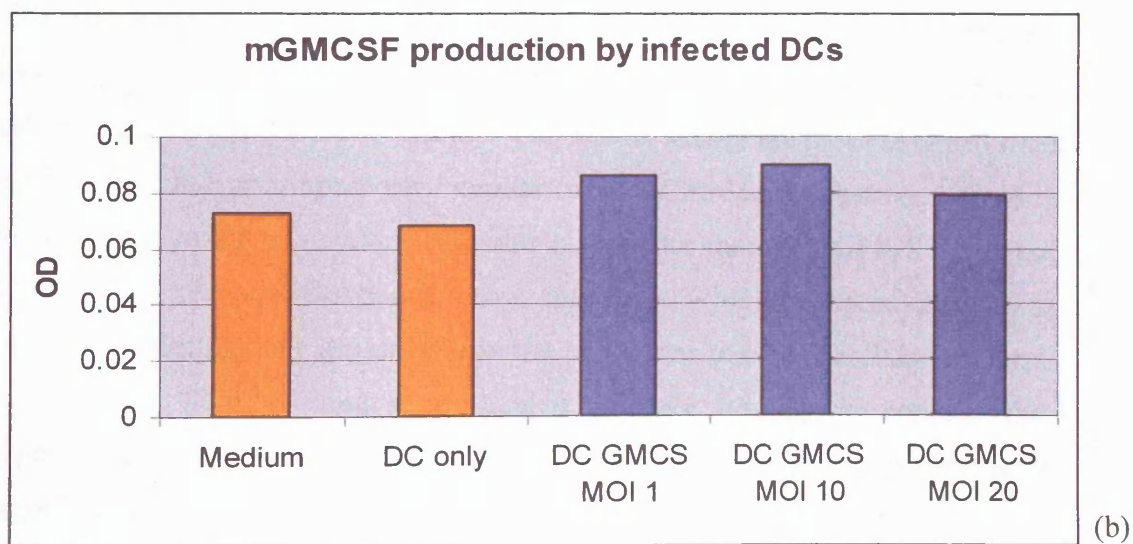
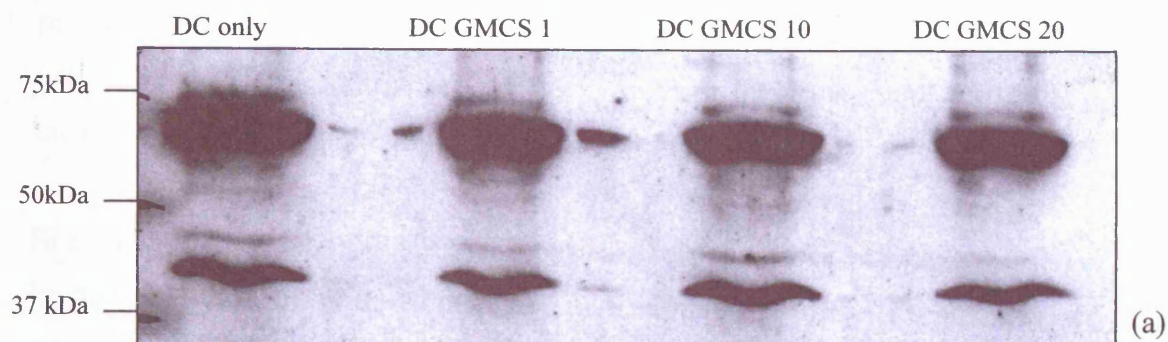


Figure 6.15: Expression of PyCS and mGMCSF in infected DCs

(a) Western blot on 10^6 non infected dendritic cells (DC only), 10^6 DC infected 24hr with $17^{+}47^{-}$ GMCSF-CS/vhs VP16 $^{-}$ at an MOI of 1 (DC GMCS 1), at an MOI of 10 (DC GMCS 10), or at an MOI of 20 (DC GMCS 20) and stained for CS.

(b) mGMCSF ELISA on the supernatant of the infected DCs. OD values are presented rather than quantitative values since these were lower than the standard curve limit.

6.3.2 Ability of 17⁺47⁻GMCSF-CS/vhsVP16⁻ to induce an IFN γ response

Immunisation with the 17⁺47⁻vhs⁻VP16⁻ backbone expressing different proteins had so far always stimulated a specific IFN γ immune response. As for the other proteins, the strength of anti-CS response was measured after immunisation of three mice per group with ex-vivo infected DC (DCV), direct virus injection (VCS), and, as a positive control, injection of DCs pulsed with the CS SYVPSAEQI CD8⁺ CTL peptide (DCCS). The negative controls received mature DC (DC only) or were not immunised. Mice were immunised both in the footpad and in the flank, and spleens were removed 14-15 days later and restimulated with peptide in vitro for 7 days. The IFN γ response was measured in an ELISPOT assay in which the spleen cells were stimulated with either A20 cells or A20 cells presenting the PyCS peptide. The values shown in figure 6.16 represent the net value obtained after subtracting the response from incubation with A20 cells to the response from incubation with A20 presenting the CS peptide.

Similarly to the anti-SSP2 experiment, no response was obtained from non immunised mice or mice immunised with mature DC only. Surprisingly, no response was obtained from two out of three mice that received neat 17⁺47⁻GMCSF-CS/vhs VP16⁻ virus, or peptide pulsed mature DCs. Two out of three mice responded to injection of infected DCs indicating that delivery of the protein may be more efficient by this method. The results shown are those obtained from one experiment, but a repeat gave similar results. Considering that co-expression of mGMCSF might give more reproducible results than if mGMCSF was not included, the responses obtained using the 17⁺47⁻GMCSF-CS/vhsVP16⁻ virus were disappointing, but do show that the virus can induce a cellular response against PyCS. Due to time restrictions it was not possible to optimise this response further, but considering the results from the PySSP2 experiments, it might have been appropriate to use larger groups of mice.

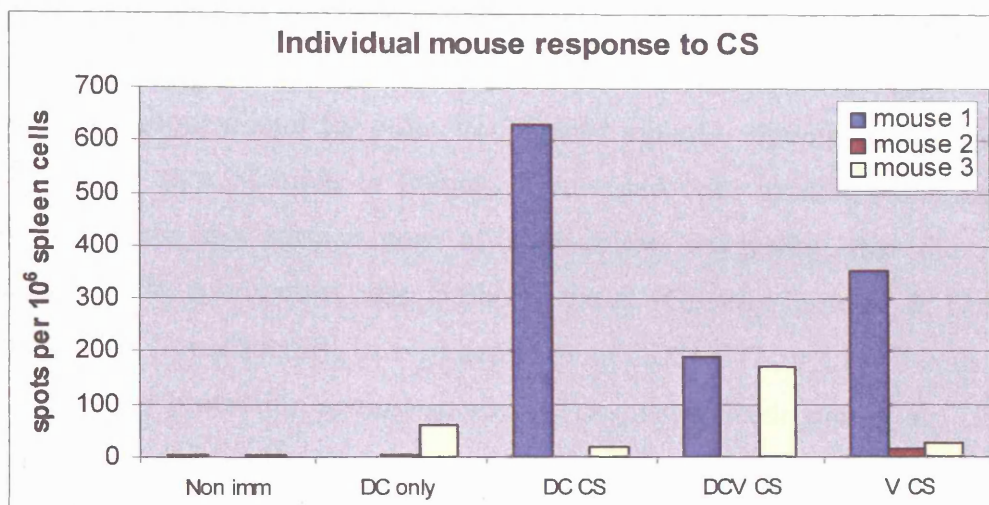


Figure 6.16: Anti-CS IFN γ response to 17⁺47-GMCSF-CS/vhsVP16⁻
 Anti-CS IFN γ ELISPOT on spleen cells from mice immunised for 2 weeks with nothing (non imm), 10⁶ mature DC (DC only), 10⁶ DC pulsed with CS peptide (DCCS), 10⁶ DC infected at MOI1 with 17⁺47-GMCSF-CS/vhsVP16⁻ (DCVCS), or with 10⁶ pfu of the virus (VCS). The response from individual mice is shown.

6.4 Discussion

The results presented in this chapter have shown that it is possible to stimulate a cellular response to the sporozoite surface proteins CS and SSP2 of *Plasmodium yoelii* by immunising with dendritic cells infected with 17⁺47⁻GMCSF-CS/vhsVP16⁻ or with 17⁺47⁻SSP2/vhsVP16⁻ or by using neat virus.

The ability to stimulate a cellular immune response against malaria proteins may be very important for vaccine development. It is now accepted that CD8⁺T cells responses are fundamental for protection against malaria, although initial studies found that the sera of mice or humans immunised with irradiated sporozoites could precipitate the surface coat of sporozoites, suggesting that the main response against sporozoites was antibody based (Oliveira-Ferreira & Daniel-Ribeiro, 2001). Subsequently, in vivo depletion of CD8⁺ CTL or CD4⁺T cells also showed reduced protection against sporozoite challenge (Rodrigues et al., 1993), whereas B cell deficient mice were shown to still be able to mount an immune response after sporozoite immunisation (Tsuji & Zavala, 2003). Additionally, transfer of cloned T cells raised against sporozoite surface proteins could give protection in mice, (Khusmith et al., 1991, Rodrigues et al., 1991, Romero et al., 1989) and treatment with anti-IFN γ antibodies in mice previously immunised with irradiated sporozoites abolished protection (Oliveira-Ferreira & Daniel-Ribeiro, 2001, Schofield et al., 1987). Thus CD8⁺ CTL cells, rather than antibodies, are the critical effector cells for protection at the pre-erythrocytic stage of malaria.

A single immunisation with the 17⁺vhs⁻47⁻VP16⁻ backbone expressing PySSP2 either as neat virus or as ex-vivo infected DCs successfully showed induction of IFN γ responses. Fortunately, no background problem was noticed in these experiments, allowing the comparison of the two methods of immunisation, and suggesting that the previous background problems were due to the peptide used. Interestingly, no significant difference in the strength of the response against PySSP2 could be seen between immunisation with infected DCs or neat virus, indicating a high efficiency of virus infection in vivo. Similar to the experiments presented here, previous studies have used DCs infected with recombinant retrovirus expressing PySSP2 to successfully induce a CTL response (Hettitihewa,

2003). Injection of neat virus expressing SSP2, has so far only been reported from MVA used as boost following DNA immunisation in mice (Schneider et al., 1998) and non human primates (Schneider et al., 2001) where protection was achieved. CTL responses to SSP2 have otherwise been induced by injection of synthetic peptides (Wang et al., 1996) or by injection of mastocytoma cells (P815) transformed with the SSP2 gene (Khusmith et al., 1991). The ability to stimulate a IFN γ response to SSP2 using the HSV1 viruses may be effective at inducing protection, as injection of CD8⁺T cell clones raised against SSP2 could induce protection in Balb/c mice (Khusmith et al., 1994).

The mechanism of action of the anti-SSP2 response has not been investigated here, although the response to SSP2 after peptide (Wang et al., 1996) or infected DC injection (Hettihewa, 2003), has been shown to involve both CTL and CD4T cells, and to take place in an IFN γ dependent mechanism. Clearance of the infected liver cells is believed to be CD8⁺CTL, IFN γ dependent (Bruna-Romero et al., 2001, Bruna-Romero & Rodriguez, 2001, Gilbert et al., 2002, Hettihewa, 2003, Li et al., 1993, Wang et al., 1996), although IFN γ independent mechanisms have also been suggested (Rodrigues et al., 2000).

It is thought that infected hepatocytes present sporozoite peptides on their surface, these are recognised and presented to CD8⁺ CTL cells which are activated and release IFN γ that induce the liver cells to eliminate the intracellular parasite by mechanisms possibly involving nitric oxide (Doolan et al., 1996). Alternatively, killing of the hepatocytes may take place in an IFN γ independent, but perforin, TNF α or Fas dependent way (Rodrigues et al., 2000). Sporozoite injection seems to trigger the IFN γ dependent pathway whereas recombinant adenovirus immunisation may rely on the alternative mechanism (Rodrigues et al., 2000). The type of response induced may be dependent on the immunising vector, HSV1, in fact, despite being a virus like adenovirus, it seems to produce results suggesting of the IFN γ dependent pathway.

Enhancers have often been used in experimental malaria vaccines, and GMCSF in particular has been shown to impair resistance to blood stage malaria (Riopol et

al., 2001). Vaccines expressing GMCSF have also been tested on non-human primates (Kumar et al., 2002) and in human clinical trials for safety (Hoffman & Doolan, 2000). GMCSF can increase the protective effect of a plasmid encoding PyCS (Weiss et al., 1998) or PbCS (Singh & Singh, 2001) when co-administered as a second plasmid, and to increase both cellular (CTL and IFN γ release) and antibody responses when included in the priming dose of a PyCSDNA-Poxvirus prime-boost immunisation (Sedegah et al., 2000). The effect that GMCSF has when expressed from HSV1, does not appear to enhance the response, but rather makes it more reproducible. Also, no significant difference was seen between injection of infected DCs or neat virus. As previously suggested in the studies looking at anti-NP responses, the lack of effect of GMCSF expression could be due to the low levels produced. Although as little as 3 μ g are enough to have an effect (Weiss et al., 1998), GMCSF may not be produced in sufficient amounts by the vectors constructed to enhance the response, although the amount present might be enough to ensure a minimal but constant recruitment of DCs and T cells.

Considering the high immunogenicity of PyCS and the additional expression of GMCSF from the vector, as well as the fact that good responses had been obtained for NP and SSP2, the highly variable responses seen from 17⁺47⁻ GMCSF-CS/vhsVP16⁻ was surprising. PyCS is a strong immunogen, the main target of the immune response upon injection of irradiated sporozoites. Many studies have used this protein either by delivering the full length gene (Rodrigues et al., 1997, Rodrigues et al., 1998, Rodrigues et al., 1994) or specific epitopes (Franke et al., 1997, Franke et al., 2000, Gonzalez-Aseguinolaza et al., 2003, Murata et al., 1996, Rodrigues et al., 1994, Tsuji et al., 1998), to successfully stimulate a response, though not necessarily protection. DCs infected with adenovirus expressing full length PyCS (AdPyCS) have been shown to stimulate both CD4⁺ and CD8⁺ T cells resulting in inhibition of liver stage parasite (Bruna-Romero & Rodriguez, 2001), and a single immunisation of Balb/c mice with adenovirus expressing PyCS (AdPyCS) can induce a protective response, involving both CD8⁺ and CD4⁺ cells (Rodrigues et al., 1997).

The strength of response to CS obtained from HSV-1 appears about ten fold lower than the number of activated T cells obtained with adenovirus, but of the same

magnitude as the response obtained by immunisation with sporozoites (Rodrigues et al., 1998), suggesting that the response seen with HSV1 expressing a single malaria protein might not be strong enough to be protective, but might be effective if multiple antigens were used simultaneously. Immunisation with single malaria antigens has in fact rarely been protective despite stimulation of both the cellular and humoral branches of the immune system, Kushmit et al (Khusmith et al., 1991) have shown that responses to both CS and SSP2 are necessary to achieve protection. Alternatively, protection against *P. yoelii* has been achieved with a prime boost regime using adenovirus and vaccinia virus expressing full length PyCS (Bruna-Romero et al., 2001) or using influenza and vaccinia virus expressing CS peptides and full length gene respectively to induce protection against *P. yoelii* (Li et al., 1993) or *P. berghei* (Gilbert et al., 2002).

Overall, confirming the results initially obtained with the influenza NP test antigen, the results from this chapter have shown that 17⁺47⁻vhs⁻VP16⁻ can be used as a backbone to stimulate cellular responses to malaria antigens. It has been possible to induce SSP2 and CS IFN γ specific responses and to show the beneficial effect of the co-expression of GMCSF in obtaining more reproducible results. The magnitude of response obtained is similar to the one reported by immunisation with sporozoites, therefore a virus co-expressing SSP2, CS and GMCSF might be a good candidate to achieve protection. Alternatively, a prime-boost regime using two different vectors expressing the same protein could be tested. In conclusion, the 17⁺vhs⁻47⁻VP16⁻ has been proved to be capable of stimulating cellular immune responses to malaria antigens both by injection of infected DCs and by injection of neat virus.

Chapter 7

Ability of HSV-1 vectors to induce antibody responses

7.1 Introduction

Dendritic cells have been investigated mainly for the stimulation of CTL responses. The experiments presented in this thesis so far, have demonstrated that the HSV-1 17⁺vhs⁻47⁻VP16⁻ backbone expressing influenza NP, plasmodium yoelii CS or SSP2 from the vhs locus, are able to induce an IFN γ response both to the virus and to the delivered protein after subcutaneous immunisation with either neat virus or ex-vivo infected DCs.

Having constructed the recombinant viruses using the full length genes, it is probable that in addition to cellular responses, a humoral response could be induced too. Expression of full length genes allows presentation of peptides both on MHCI and MHCII molecules. Therefore any epitope, including CD4⁺T cells and B cell epitopes can be presented. Although DCs are known for their unique ability to prime naïve T cells, they can also activate B cells directly (Banchereau et al., 2000, Kikuchi et al., 2000), or indirectly via CD4T cells. Immunisation with DCs infected with recombinant HSV1 vectors therefore offer the possibility of stimulating both humoral and cellular immune responses.

In the construction of a vaccine vector, and for malaria in particular where both intracellular and extracellular stages are present, the possibility of stimulating both arms of the immune system would provide a significant advantage. Especially when using HSV1, in which multiple genes can be inserted inducing both types of response, multiple stages of an infection can be targeted. With regard to malaria, antibodies should be able to block hepatocyte infection by sporozoites, thus avoiding merozoite release by infected liver cells. Similarly, antibodies targeted to merozoites would inhibit red blood cell infection and the onset of clinical malaria.

Although cellular responses seem to play the dominant role in combating malaria infection, antibodies have also been shown to be important. Initial observations showed that mice immunised with irradiated sporozoites produced, in addition to CTL responses, anti-sporozoites antibodies (Charoenvit et al., 1987, Rodrigues et al., 1998), in particular against PySSP2 (Charoenvit et al., 1987) and PyCS. Also,

passive transfer of monoclonal antibodies against the CS protein could protect mice against malaria (Charoenvit et al., 1991b), and passive transfer of sera from immune African individuals to Thai children resulted in protection (Sabchareon et al., 1991). Recent studies have also highlighted the importance of antibody responses to multiple malaria antigens using oral immunisation of mice and showing that protection was dependent on the induction of antibody responses (Wang et al., 2003). Finally, strong evidence of the importance of antibody responses in malaria therapy has been provided by a recent vaccine clinical trial (RTS,S/AS02) which uses the carboxy terminal of the CS protein fused to the hepB surface antigen (HbsAg) expressed together with unfused HbsAg in yeast as the immunogen (Stoute et al., 1997). This was administered with an adjuvant (AS02) and showed protection (Kester et al., 2001) correlated with strong antibody and cellular immune responses (Bojang et al., 2001, Lalvani et al., 1999).

Viral vectors have the ability to induce both cellular and humoral responses, but protection may not necessarily require both of these (Rodrigues et al., 1997). Adenovirus expressing PyCS (AdPyCS) has been shown to be able to induce antibody responses to the protein in addition to stimulation of a strong cellular response (Rodrigues et al., 1998). Similarly, both CTL and antibodies to CS have been reported after immunisation with recombinant vaccinia or pseudo-rabies virus expressing CS (Khusmith et al., 1991), although protection was not achieved.

The CS protein binds to hepatocytes (Frevert et al., 1993), and there is evidence that in addition to CD8⁺T cells, antibodies to the central repeat region of *P. yoelii* CS, which contain the immunodominant B cell epitope mediates protection against malaria (Charoenvit et al., 1991b, Marussig et al., 1997). Antibodies against the repeat region are able to immobilise sporozoites (Stewart et al., 1986), and neutralise their infectivity (Gantt et al., 2000). It was therefore thought that it would be beneficial to stimulate antibody responses to this protein. Studies have shown that the inclusion of MHCII CD4⁺T cells epitopes is necessary to induce high levels of antibodies against the repeat region of CS (Tsuji & Zavala, 2003), and expression and presentation of both T and B epitopes from the same protein should facilitate natural boosting (Bharadwaj et al., 1998). Having included full

length antigens within the vectors constructed in this thesis, it would be likely that in addition to the induction of an IFN γ response, antibody responses might also be induced.

For SSP2, which is known to play a central role in cell invasion, point mutations in the metal ion-dependent adhesion site greatly impair the ability of sporozoites to invade hepatocytes (Michishita et al., 1993), suggesting that again, antibodies against this protein might be beneficial. Although in some cases, a certain degree of inhibition of hepatocyte invasion has been achieved (Charoenvit et al., 1997, Rogers et al., 1992), antibodies against SSP2 have been suggested to be of low effectiveness against sporozoite invasion (Gantt et al., 2000). Passive immunisation experiments using high concentrations of antibodies against SSP2 repeats very rarely showed protection against sporozoite challenge (Charoenvit et al., 1987, Rogers et al., 1992, Wang et al., 1996), and no protection was achieved either when high titres of anti-SSP2 antibodies against undetermined epitopes were induced (Khusmith et al., 1991). Nonetheless, being able to stimulate antibody responses to various antigens may well be important. This has been shown in monkeys, where beneficial antibody responses were stimulated to both the preerythrocytic and erythrocytic stages of malaria (Rogers et al., 2002) by immunisation with DNA and poxvirus expressing proteins from different stages of the malaria life cycle together with GMCSF.

The inclusion of GMCSF in a malaria vaccine has previously been shown to improve the level of protection induced by a plasmid vaccine by increasing both CD4 and CD8 responses and also enhancing the level of antibody produced against PyCS (Weiss et al., 1998). Also, the addition of GMCSF has been shown to increase the antibody responses induced by expression of full length PyCS gene (Weiss et al., 1998). As a vaccine able to combine both immune effector mechanisms is more likely to be efficient in protecting against infection, it was therefore decided to investigate the ability of vectors constructed to induce antibody responses to the delivered proteins, and to the virus, and to assess the effect of co-expressing GMCSF.

Viruses used in this chapter

This diagram indicates the progression of virus construction
Specific deletions and inactivations of each backbone are specified

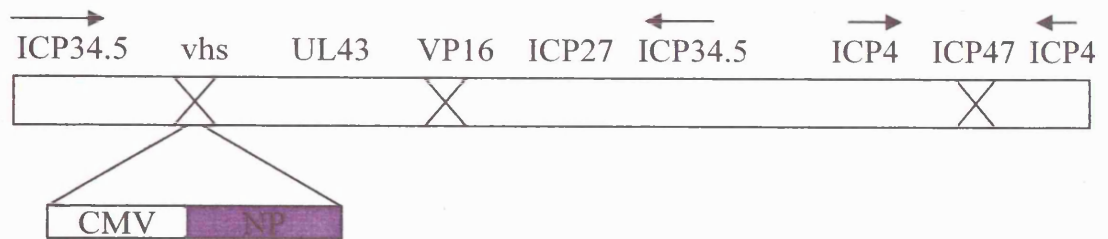
X indicates inactivity of the gene either by deletion or inactivation
→ indicates the direction of gene expression

17⁺47⁻NP/vhsVP16⁻

ICP47 gene deleted

vhs gene inactivated by insertion of the CMV-NP cassette

VP16 gene with V422 mutation

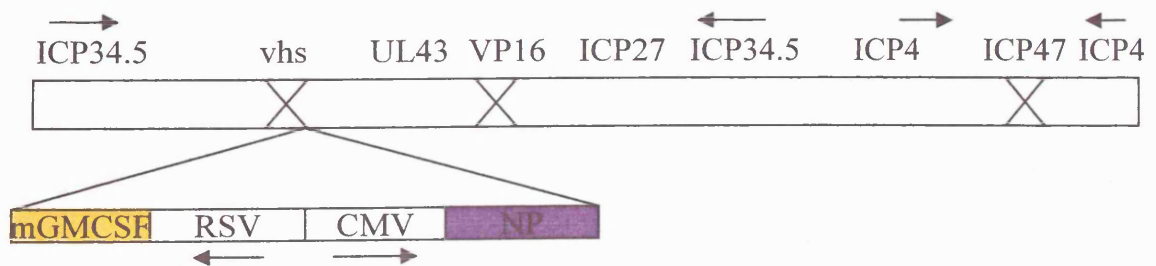


17⁺47⁻mGMCSF-NP/vhsVP16⁻

ICP47 gene deleted

vhs gene inactivated by insertion mutation of the mGMCSF-NP cassette

VP16 gene with V422 mutation

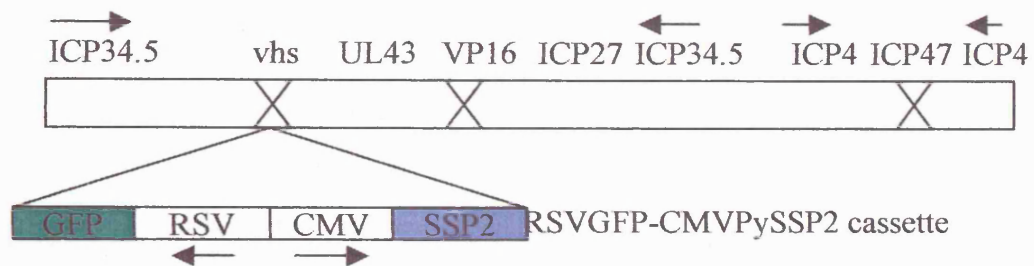


17⁺47⁻ RSVGFP-CMVSSP2/vhsVP16⁻

ICP47 gene deleted

vhs gene inactivated by insertion of the RSVGFP-CMVPySSP2 cassette

VP16 gene with V422 mutation

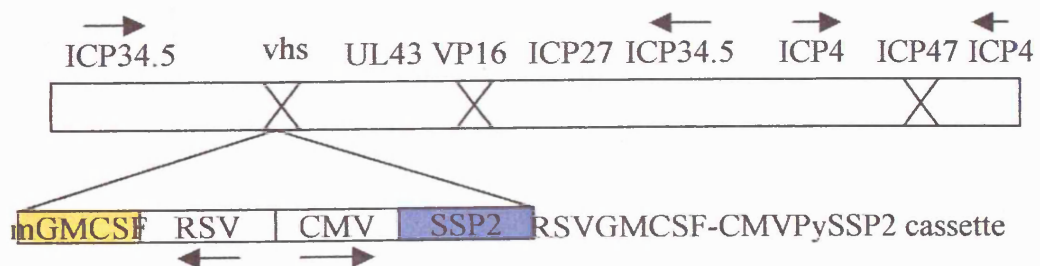


17⁺47⁻ RSVGMCsf-CMVSSP2/vhsVP16⁻

ICP47 gene deleted

vhs gene inactivated by insertion of the RSVGMCsf-CMVPySSP2 cassette

VP16 gene with V422 mutation

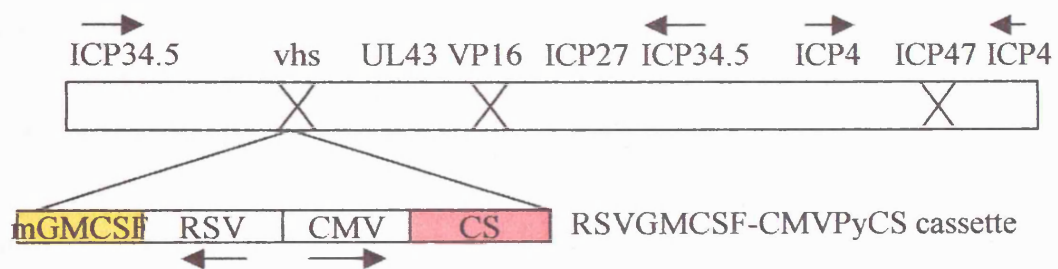


17⁺47⁻ RSVGMCSE-CMVCS/vhsVP16⁻

ICP47 gene deleted

vhs gene inactivated by insertion of the RSVGMCSE-CMVPyCS cassette

VP16 gene with V422 mutation



7.2 Antibody response to NP after immunisation with 17⁺47⁻NP/vhs VP16⁻ or 17⁺47⁻GMCSFNP/vhs VP16⁻.

The results in this thesis have shown that it is possible to induce an IFN γ response to both the delivered antigen and to the virus vector two weeks after immunisation when using the 17⁺vhs⁻47⁻VP16⁻ backbone. Depending on the pathology of interest and on the antigen, it may be beneficial to also induce a humoral response. It was therefore decided to determine the antibody responses induced by immunisation with 17⁺47⁻NP/vhs VP16⁻ and with 17⁺47⁻GMCSFNP/vhs VP16⁻.

7.2.1.Optimisation of the anti- NP antibody ELISA

The anti-NP antibody ELISA assay was optimised using different concentrations of purified NP protein to coat microtitre plates to identify the amount of NP allowing maximal sensitivity without non specific secondary antibody binding. A range of 1000ng-5ng of purified protein was tested both with and without the primary antibody to check for non-specific binding of secondary antibody (figure 7.1). When coating with 40ng of NP, maximal OD was achieved with minimal secondary antibody binding (figure 7.1). This concentration was therefore used to coat plates to carry out assays on serum samples.

7.2.2 Detection of anti-NP antibodies in serum samples

The mice used for this experiment were those used for the experiment comparing the effect of GMCSF expression (§ 5.3.4). The groups of mice were non immunised, or received mature DC (DC only), mature DC loaded with NP peptide (DC NP), DC infected with 17⁺47⁻NP/vhs VP16⁻ (DCV), DC infected with GMCSF-NP virus (DCGM), neat NP virus (V), or neat GMCSF-NP virus (VGM). Serum from mice two weeks post-immunisation was tested for presence of anti-NP antibodies at the dilution (1:10) at which the OD value from non immunised mice and mice immunised with mature DC only was in the same range as the negative control (no serum) (figure 7.2). No difference could be seen between the groups (figure 7.2), implying that there was no anti-NP antibody response generated. The possible reasons for this will be discussed later.

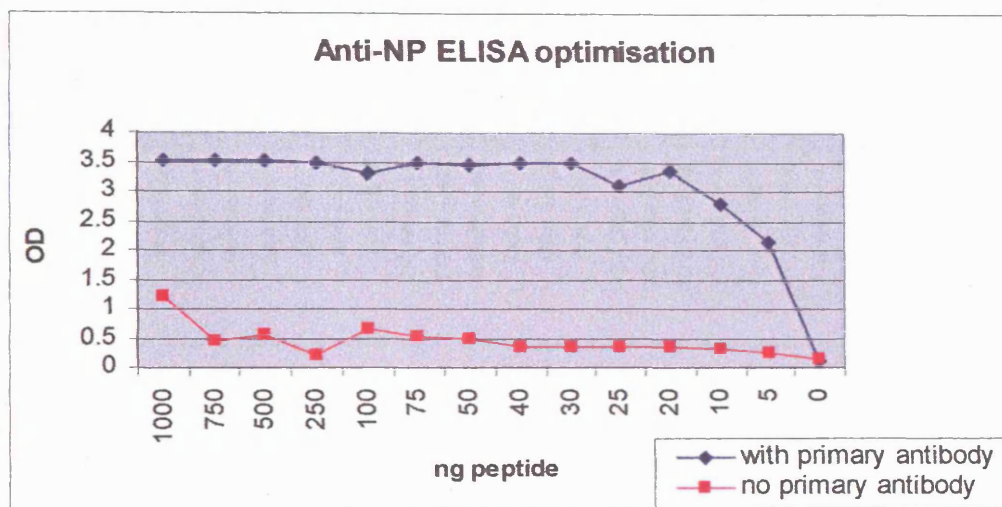


Figure 7.1: Anti-NP antibody ELISA optimisation

The ELISA test was optimised for the coating protein using a range (5ng-1 μ g) of purified NP and using a constant amount of commercial primary anti-NP antibody and secondary antibody (blue line, with primary controls).

The different amounts of protein were also tested for non specific binding of secondary antibody by carrying out the test omitting the primary antibody (pink line, secondary antibody only).

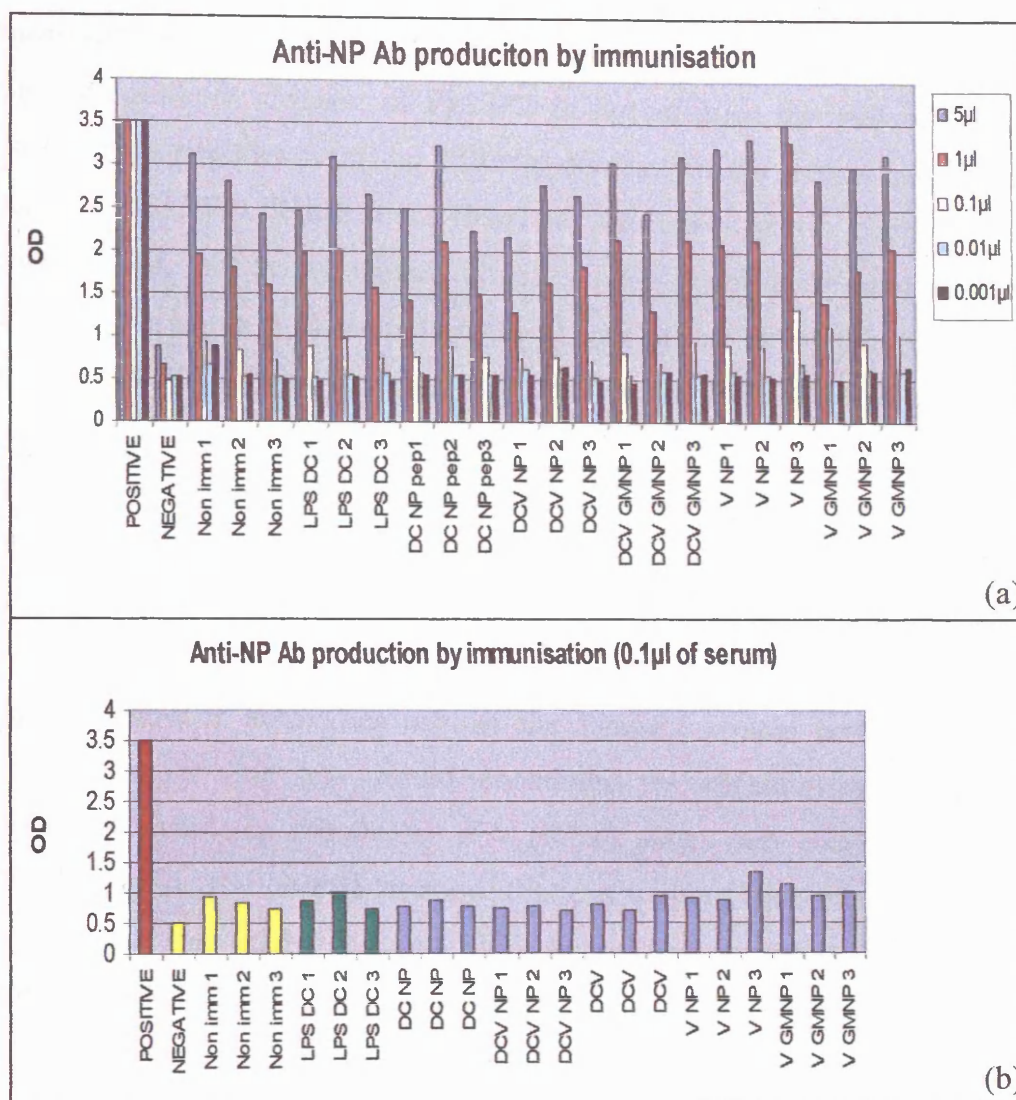


Figure 7.2: Anti-NP antibody response

ELISA test showing the production of anti-NP antibodies in the serum of mice 2 weeks after immunisation with nothing (non immunised), mature DC (LPS DC), mature DC pulsed with NP peptide (DC NP), DC infected at MOI of 1 with 17⁺47-CMV-NP/vhsVP16⁻ (DCVNP), or with 17⁺47-GMCSF-NP/vhs VP16⁻ (DCVGMNP), or with 10⁶pfu of neat 17⁺47-CMV-NP/vhsVP16⁻ (VNP) or neat 17⁺47 - GMCSF-NP/vhs VP16⁻ (VGMNP). The positive control is the value obtained by using commercial anti-NP antibody instead of serum and the negative control is the value obtained by using medium instead of serum.

Various dilutions of the serum were tested (a) and the dilution where the value of the negative control was similar to the non immunised mice (0.1µl of serum) was used for comparison of the groups (b).

7.3 Antibody response to PySSP2 after immunisation with 17⁺47⁻GFP-SSP2/vhs VP16⁻ or 17⁺47⁻GMCSF-SSP2/vhs VP16⁻

7.3.1 Optimisation of the anti-SSP2 antibody ELISA

The protective antibody domain of PySSP2 in Balb/c mice has still not been identified. Various peptides from the different domains of the protein have been tested (Gantt et al., 2000, Wang et al., 1996) but it has been shown, contrarily to what was expected, that the immunodominant domain is not in the extracellular repeat domain, nor in the A domain or in the N terminus of the protein (Gantt et al., 2000). Despite the fact that monoclonal antibodies against different peptides of the SSP2 repeats do not inhibit in vivo infectivity of *P. yoelii* sporozoites in Balb/c mice (Gantt et al., 2000, Wang et al., 1996), the (NPNEPS)₃ peptide was used in the experiments presented here for the detection of anti-SSP2 antibodies. Antibodies against this peptide alone are not protective in Balb/c though they are effective in A/J mice at protecting (Wang et al., 1996). However, using this peptide should show if antibodies against the PySSP2 protein are produced. Positive results from this test would encourage investigation of antibody production against whole sporozoites by IFA. ELISA plates were coated with the PySSP2 B cell peptide (NPNEPS)₃ at concentrations varying between 750ng and 10ng. To optimise the assay, the anti-SSP2 hybridoma supernatant antibody (F3B5, gift from Dr A.Rodriguez) was used as the primary antibody and the different peptide concentrations were tested both with and without this. The background staining of secondary antibody is very low even at high peptide concentrations. The optimal coating dilution was therefore determined with regard to the detection sensitivity. When using 50ng of peptide, the OD starts to drop slightly (figure 7.3). It was therefore decided to use 60ng of peptide per well which gave optimal results with minimal secondary antibody binding.

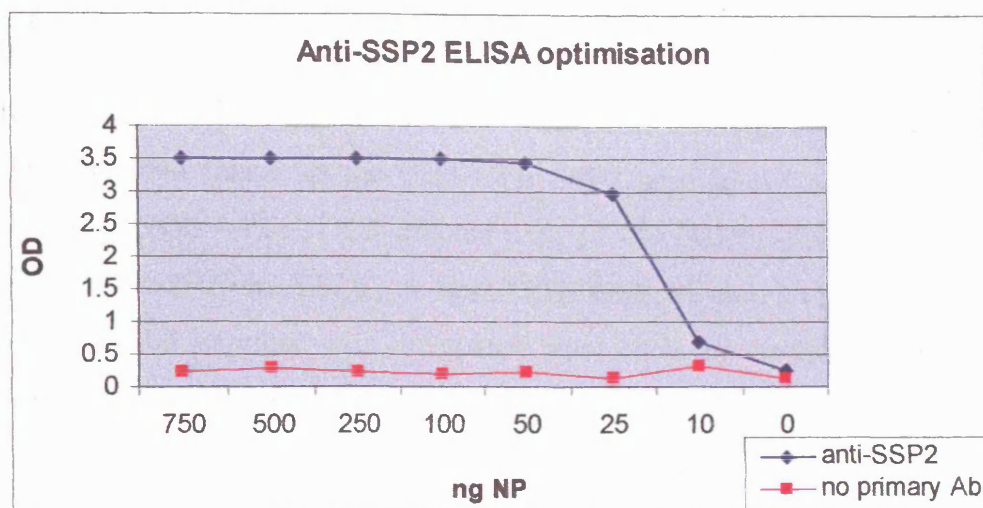


Figure 7.3: Anti-SSP2 antibody ELISA optimisation

The ELISA test was optimised for the coating peptide using a range (10ng-750ng) of SSP2 (NPNEPS)₃ peptide and using a constant amount of primary anti-SSP2 antibody and secondary antibody (blue line, anti-SPP2).

The different amounts of coating peptide were also tested for non specific binding of secondary antibody by carrying out the test omitting the primary antibody (pink line, no primary Ab).

7.3.2 Detection of anti-SSP2 Ab in serum samples of mice two weeks after immunisation

The antibody response induced by immunisation with the different viruses was investigated by performing a sandwich ELISA assay on serum samples of immunised mice. The sera from the same mice that had previously been tested for IFN γ responses (§6.2.3) were analysed for the presence of anti-SSP2 antibodies. Three mice per group had been immunised with either nothing, LPS mature DC, mature DC that had taken up the CD8⁺CTL SSP2 peptide, DCs infected with 17⁺47⁻ GFP-SSP2/vhs VP16⁻, DCs infected with 17⁺47⁻GMCSF-SSP2/vhs VP16⁻, neat 17⁺47⁻ GFP-SSP2/vhs VP16⁻, or neat 17⁺47⁻GMCSF-SSP2/vhs VP16⁻. The serum was removed together with the spleen from each mouse two weeks after immunisation and tested for presence of anti-SSP2 antibodies (figure 7.4).

Similarly to the experiment carried out for the NP expressing viruses, different dilutions of serum were tested (figure 7.4a). The signal obtained was very low at all concentrations used. When testing 1 μ l of serum, the values obtained from non immunised mice and mice immunised with mature DC were similar to the negative control (no serum). These were therefore considered further (figure 7.4b), but unfortunately no difference could be seen between the different groups (figure 7.4b). As for the IFN γ response, the effect of GMCSF on the production of anti-SSP2 antibodies was investigated using slightly larger groups for immunisation with neat virus (figure 7.5). Contrarily to the IFN γ responses, no difference could be attributed to GMCSF, suggesting that no antibody response is produced after two weeks immunisation with the 17⁺47⁻ GFP-SSP2/vhs VP16⁻ or 17⁺47⁻GMCSF-SSP2/vhs VP16⁻ viruses, as was the case for NP. Strangely two high responses appeared at 0.01 μ l and 0.001 μ l of the vGMSSP2 sample, this can probably be attributed to handling of the sample during dilution of the samples.

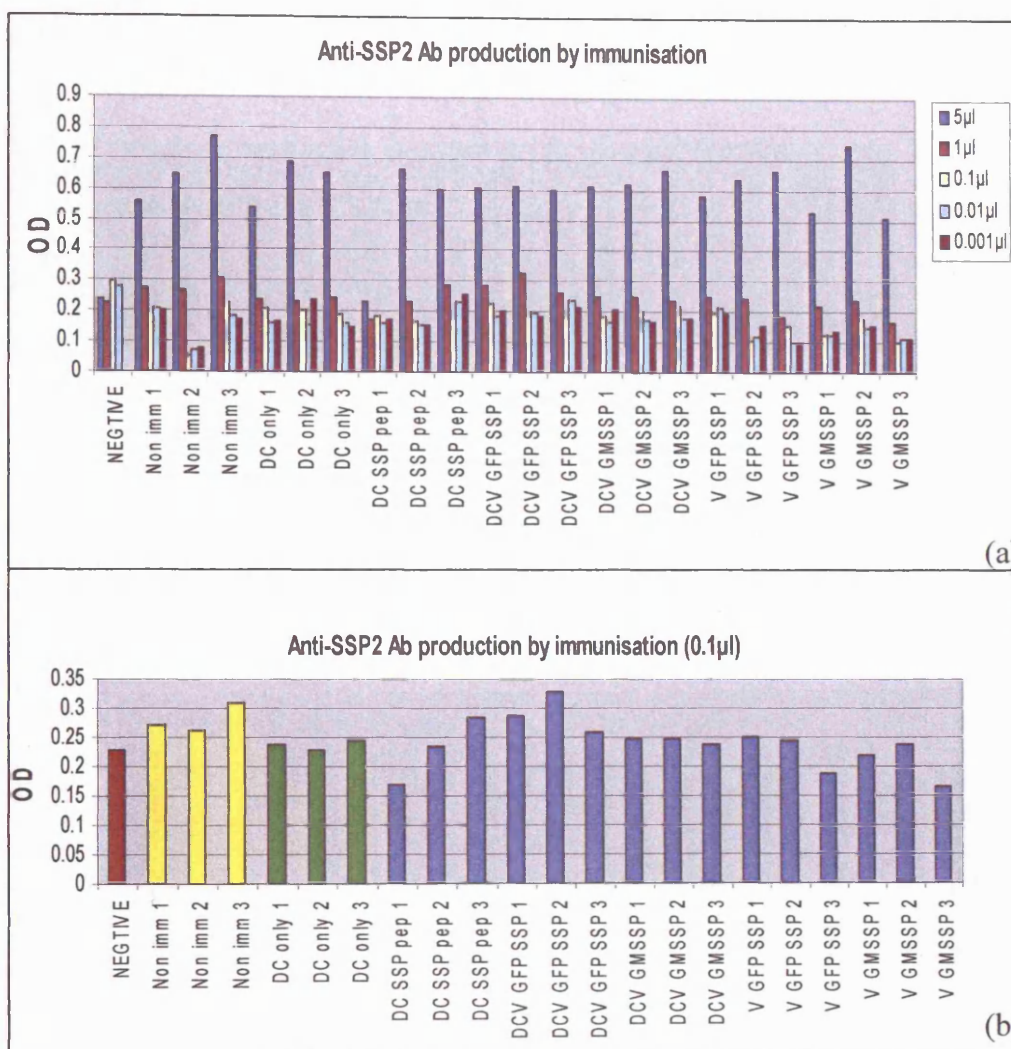


Figure 7.4: Anti-SSP2 antibody responses

ELISA test to detect anti-SSP2 antibodies in the serum of mice 2 weeks after immunisation with nothing (non imm), mature DC (DC only), mature DC pulsed with SSP2 peptide (DC SSP pep), DC infected at MOI of 1 with 17^{+47} -GFP-SSP/vhsVP16⁻ (DCVGFPSSP), or 17^{+47} -GMCSF-SSP/vhs VP16⁻ (DCVGMSSP), or with 10^6 pfu of neat 17^{+47} -GFP-SSP/vhsVP16⁻ (VGFPSSP) or neat 17^{+47} -GMCSF-SSP/vhs VP16⁻ (VGMSSP). The negative control is the value obtained by using medium instead of serum.

Various amounts of the serum were tested (a) and that in which the negative control was similar to the non immunised mice (0.1 µl of serum) was used for comparison of the groups (b).

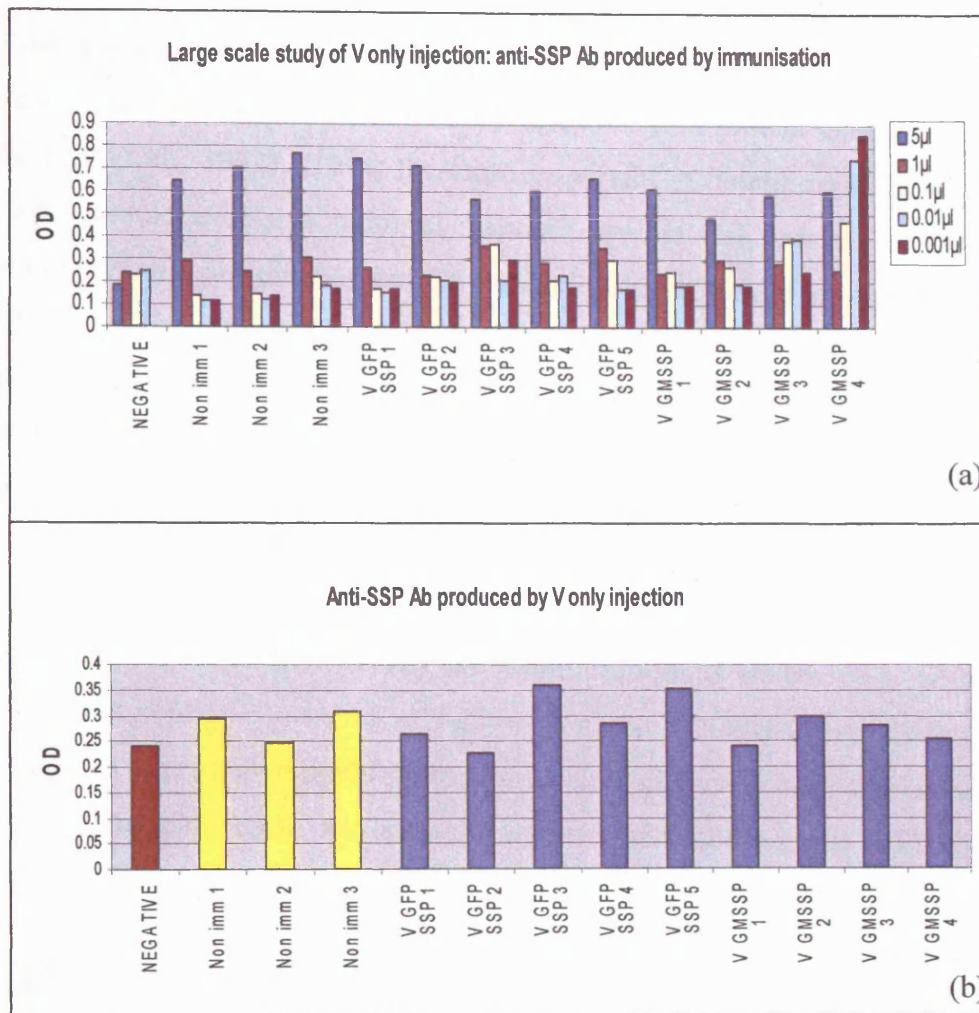


Figure 7.5: The effect of GMCSF on antibody responses to SSP2

Anti-SSP2 ELISA using serum from non immunised mice (yellow) or mice immunised 2 weeks previously with 10^6 pfu of 17^{+47} -GFP-SSP/vhsVP16⁻ (VGFPSSP) or 17^{+47} -GMCSF-SSP/vhs VP16⁻ (VGMSSP). (a) Various amounts were tested and compared to the negative control (media alone). (b) The amount of serum at which non immunised mice give values similar to the negative control (1µl) was used to compare the different groups.

7.4 Antibody response to PyCS after immunisation with 17⁺47GMCSF-CS/vhs VP16⁻

7.4.1 Optimisation of the anti-CS ELISA assay

Passive transfer of monoclonal antibodies against PyCS protect animals against malaria, (Ak et al., 1993). It was therefore important to determine if the viruses constructed would induce a humoral response against this protein. As for the previous assays, the presence of anti-CS antibodies in the sera of immunised mice was determined by sandwich ELISA. Plates were coated with the B cell immunodominant peptide of PyCS (QGPGAP)₂. The B cell epitope of PyCS has been mapped to the repeat region of the protein. The (QGPGAP)₂ peptide has been extensively studied and used for immunisation or in ELISA for identification of antibodies in the serum (Charoenvit et al., 1991b). Being able to determine the presence of antibodies against this peptide may provide an insight into protective immunity (Charoenvit et al., 1991b), as immunisation of Balb/c mice with a multiple antigen peptide containing a T epitope and three QGPGAP repeats as a B cell epitope has previously shown protection against challenge with sporozoites. Here the level of anti-repeat antibodies correlated with anti-parasite antibodies and protection. Protection did not depend upon CD4 or CD8 cells (Marussig et al., 1997). In addition, passive transfer of antibodies against the (QGPGAP)₂ region were shown to induce protection, therefore indicating that this region is an important target of antibodies (Ak et al., 1993).

To optimise the anti-CS ELISA assay, microtitre plates were coated with various dilutions of the peptide to determine the optimum coating concentration that would allow maximal antibody detection with minimal non specific binding of secondary antibody. Peptide concentrations were tested in a range from 1-1000ng (figure 7.6). Similarly, the 9D3 anti-CS antibody raised against this peptide and used in the assay (gift from Dr Tsuji) was tested at varying dilutions.

It appeared that the maximal level of detection that could be obtained was relatively low, only reaching OD 1.3. Using higher concentrations of antibody did not improve detection of the peptide but resulted in even higher background when no peptide was present. It was decided to use the antibody at 1:50 since the

purpose of the experiment was as a positive control to ensure the functionality of the assay, regardless of background staining, and lower concentrations of antibodies strongly decreased the level of detection without considerably decreasing the background. Coating of the wells using 750ng/well gave very low signal when no antibody was added, but a strong signal in presence of antibody and was used for the assay as described below.

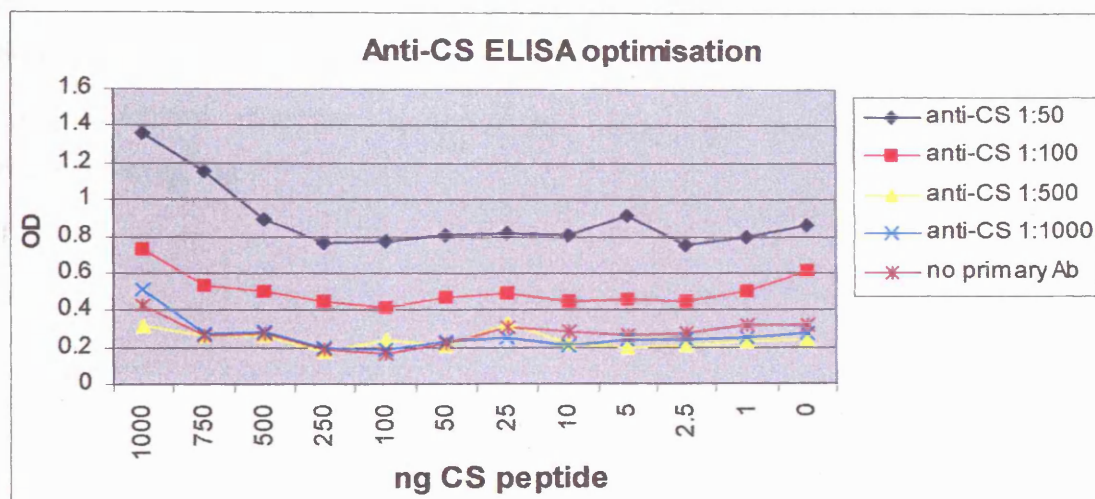


Figure 7.6: Anti-CS antibody ELISA optimisation

The ELISA test was optimised for the coating peptide using a range (1ng-1µg) of CS (QGPGAP)₃ peptide and for the primary antibody 9D3 using a range of dilutions (1:50-1:1000) but using constant amounts of secondary antibody.

The different amounts of coating peptide were also tested for non specific binding of secondary antibody by carrying out the test omitting the primary antibody (purple line, no primary Ab).

7.4.2 Measurement of anti-CS antibody level in serum two weeks after immunisation

Sera were collected from mice two weeks after immunisation with either nothing, mature DC, mature DCs loaded with the CS CD8⁺ CTL peptide, DC infected ex-vivo with 17⁺47⁻GMCSF-CS/vhs VP16⁻, or with 17⁺47⁻GMCSF-CS/vhs VP16⁻ directly. Serial dilutions of the sera were tested for presence of anti-CS antibodies (figure 7.7). However, as before, none of the groups showed detectable antibody levels looking at 1:10 dilution (figure 7.7). Considering that no response could be obtained from immunisation with the viruses expressing NP or SSP2 either, the inability to detect antibody production in this experiment is unlikely to be due to the low maximal detection level of the assay, but possibly due to the immunisation regime.

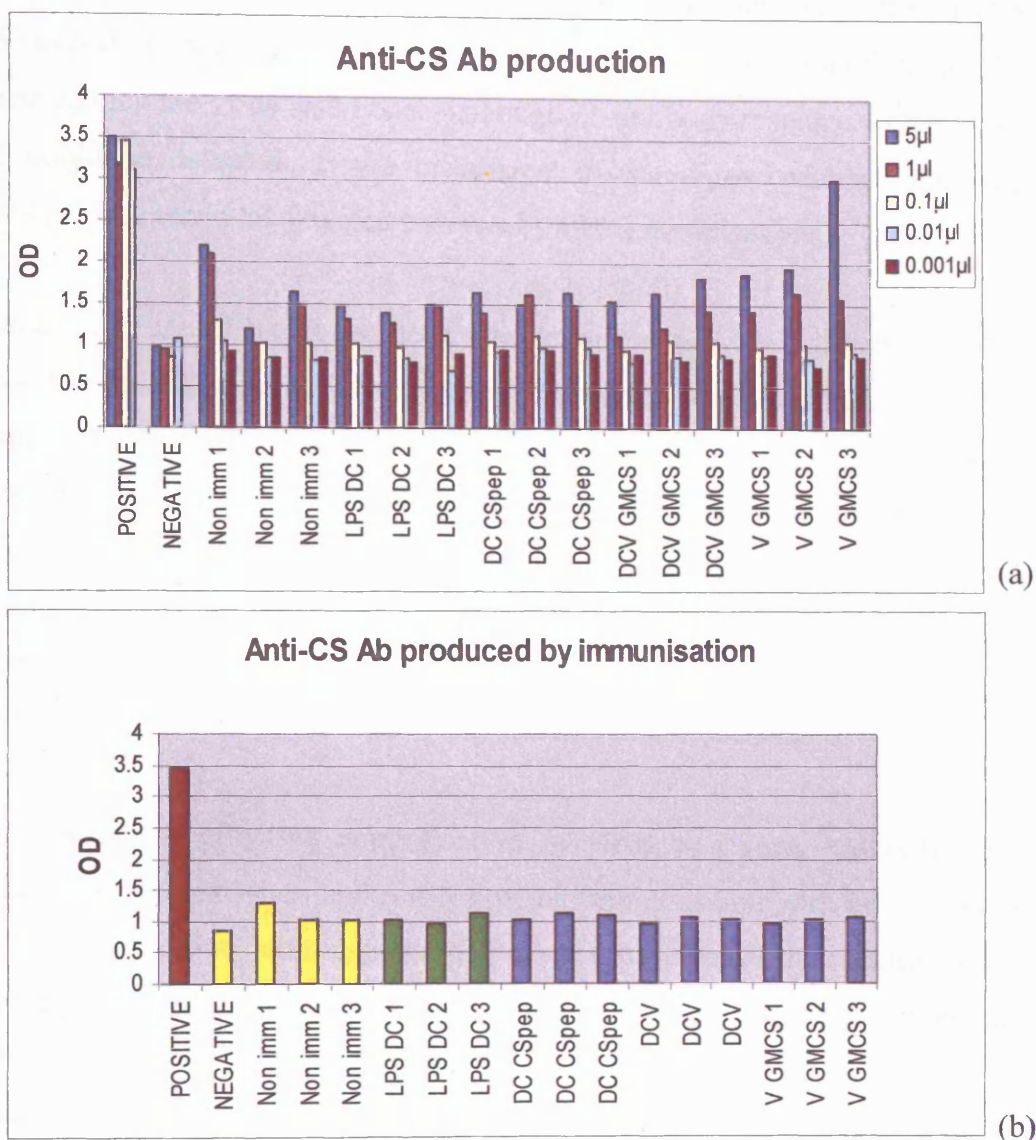


Figure 7.7: anti-CS antibody response

ELISA test showing the production of anti-CS antibodies in the serum of mice 2 weeks after immunisation with nothing (non imm), mature DC (LPS DC), mature DC pulsed with CS peptide (DC CSep), DC infected at MOI of 1 with 17⁺47-GMCSF-CS/vhsVP16⁻ (DCV), or with 10⁶pfu of neat 17⁺47-GMCSF-CS/vhsVP16⁻ (VGMCS). The positive control is the value obtained by using anti-CS antibody instead of serum and the negative control is the value obtained by using medium instead of serum.

Various amounts of the serum were tested (a) and the amount at which values from of the negative control and the non immunised mice were similar (0.1 μ l) was used for comparison of the groups (b).

7.5 Antibody responses to HSV-1

Previous results have shown (§ 5.2.2.2, 5.2.3.3) that following neat virus injection as well as following injection of infected DCs, there is stimulation of strong IFN γ response against the virus backbone. Although no antibody response to NP, SSP2, or CS could be detected, it was of interest to investigate whether antibodies against the virus could be detected two weeks after immunisation.

The same serum samples that had been used to look at anti-NP antibody responses were used in an HSV1 neutralisation assay. The assay consists (figure 7.8) of the incubation of serum samples with a known amount virus (17^{+47} NP/vhsVP16 $^{-}$) and this was then plated on cells that allow virus growth and the titre of the virus then counted. All samples were titrated in duplicate. If antibodies against the virus were present in the serum, these should neutralise the free virus resulting in a decrease in titre. Alternatively, if immunisation had not produced antibodies against the virus the titre of the input virus should remain unchanged.

In this experiment, effective neutralisation of virus was seen, but only after immunisation with neat virus and not following immunisation with infected DCs (figure 7.9). In addition, although the differences were small, the neutralisation appeared to be slightly enhanced in mice immunised with the virus expressing GMCSF.

These results therefore show that antibodies are being produced against the virus, although the detected antibody levels were not increased. That antibodies did not appear to also have developed against the delivered protein is perhaps not entirely surprising as the antibody response against all viral epitopes of all viral structural proteins are effectively being tested here, whereas antibodies against only one epitope were investigated for the responses to NP, SSP2 or CS. Since the anti-HSV response is only obtained following neat virus injection, it seems possible that there is predominant infection of cells other than DCs.

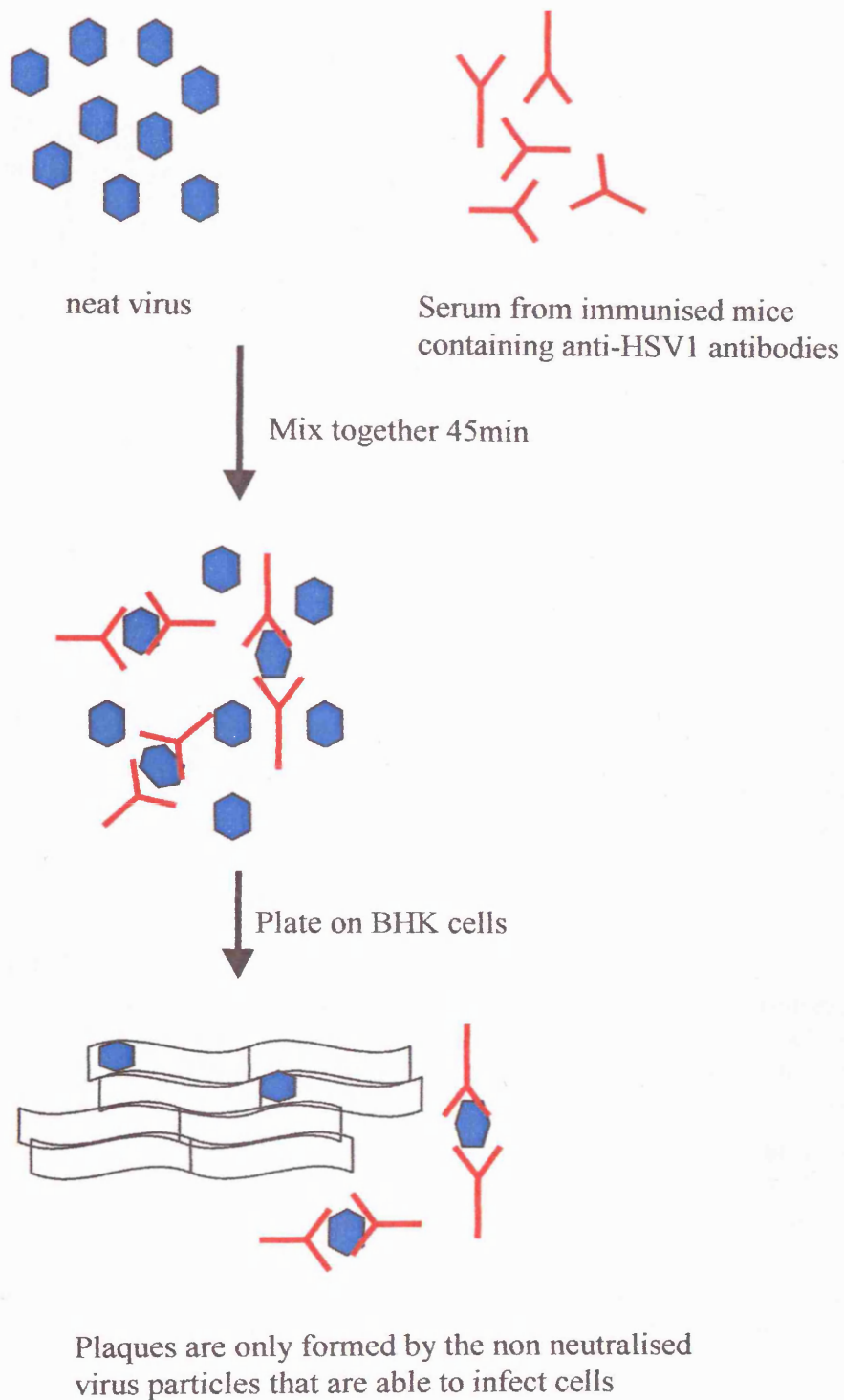


Figure 7.8: Schematic representation of neutralisation assay

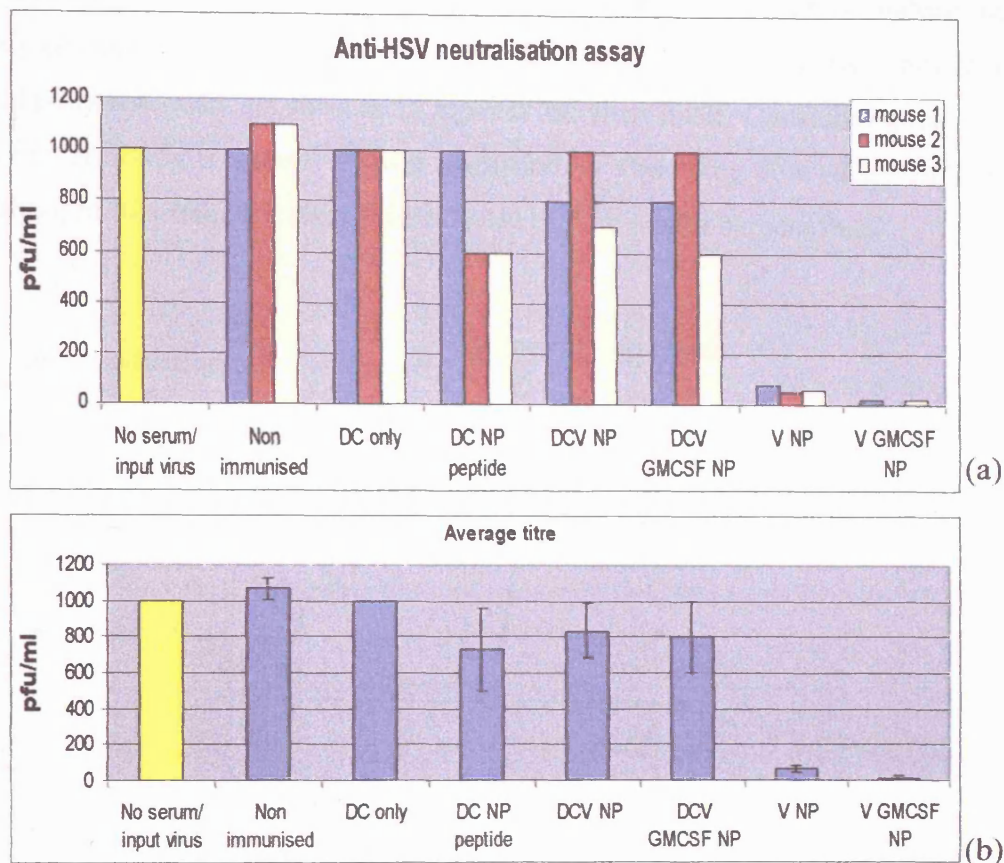


Figure 7.9: Anti-HSV antibody response

HSV-1 neutralisation assay using serum samples from mice immunised 2 weeks previously with nothing (non immunised), mature DC (DC only), mature DC pulsed with NP peptide (DC NPpeptide), DC infected at MOI of 1 with 17^{+47} -CMV-NP/vhsVP16⁻ (DCVNP), 17^{+47} -GMCSF-NP/vhs VP16⁻ (DCVGMNP) or with 10^6 pfu of neat 17^{+47} -CMV-NP/vhsVP16⁻ (VNP) or neat 17^{+47} -GMCSF-NP/vhs VP16⁻ (VGMNP). Serum was incubated with 17^{+47} -CMV-NP/vhsVP16⁻ and then plated on BHK cells for plaque formation. The groups are compared to the sample in which no serum was added (no serum/input virus) confirming the initial amount of input virus. The responses from individual mice (a) or the average of the groups (b) are shown.

7.6 Effect of boosting for two weeks on the induction of antibody responses

7.6.1 Effect of boosting on the induction of the antibody response to NP

The previous results suggested that two weeks were not enough to induce an antibody response against the recombinant protein carried by the virus although a humoral response could be stimulated against the virus itself. Considering that the onset of an antibody response is much amplified by a boosting dose (diagram 7.1), it was thought that undertaking a prime-boost regime would be beneficial.

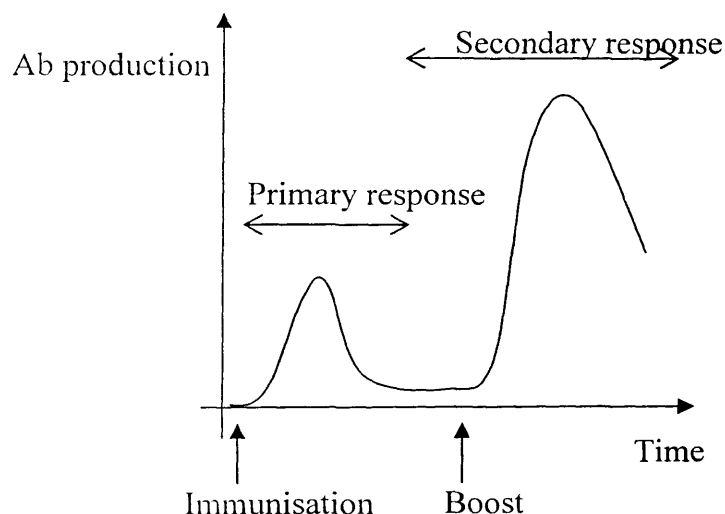


Diagram 7.1 Representation of the dynamics of an antibody response.

The effect of boosting was only investigated on the serum form mice immunised with neat virus. 10^6 pfu of $17^{+}47^{-}$ GMCSFNP/vhs $VP16^{-}$ were injected in the flank and footpad of mice, using three mice per group. Non immunised mice were kept as controls. Two weeks after the first immunisation, mice received an identical boost immunisation and were then sacrificed two weeks later. All three mice that had received the virus showed a convincing antibody response (figure 7.10). Boosting therefore gave an advantage for the induction of a humoral response against the recombinant protein carried by the virus.

7.6.2 Effect of boosting on the induction of the antibody response to PySSP2

Using the 17⁺47⁻GFP-SSP2/vhs VP16⁻ and the 17⁺47⁻GMCSF-SSP2/vhs VP16⁻ viruses, it was possible to look both at the effect of boosting on the stimulation of anti-SSP2 Ab responses and at the effect of GMCSF on the induction of this type of response. Three mice per group received flank and footpad injections of 10⁶ pfu of either viruses and two weeks after the first injection a second dose was administered for an additional two weeks before collecting the sera.

Serial dilutions of the serum from each immunised or non immunised mouse were tested for presence of anti-SSP2 Ab where it appeared that both mice immunised with 17⁺47⁻GFP-SSP2/vhs VP16⁻ or 17⁺47⁻GMCSF-SSP2/vhs VP16⁻ were able to produce anti-SSP2 antibodies. The values obtained from non immunised mice were slightly higher than the negative control (no serum) but even when the dilution at which these controls gave similar values (0.1 µl) it was possible to see a clear positive result in immunised mice.

Comparing the two viruses used is possible to conclude that expression of GMCSF does not induce stronger antibody responses in this case.

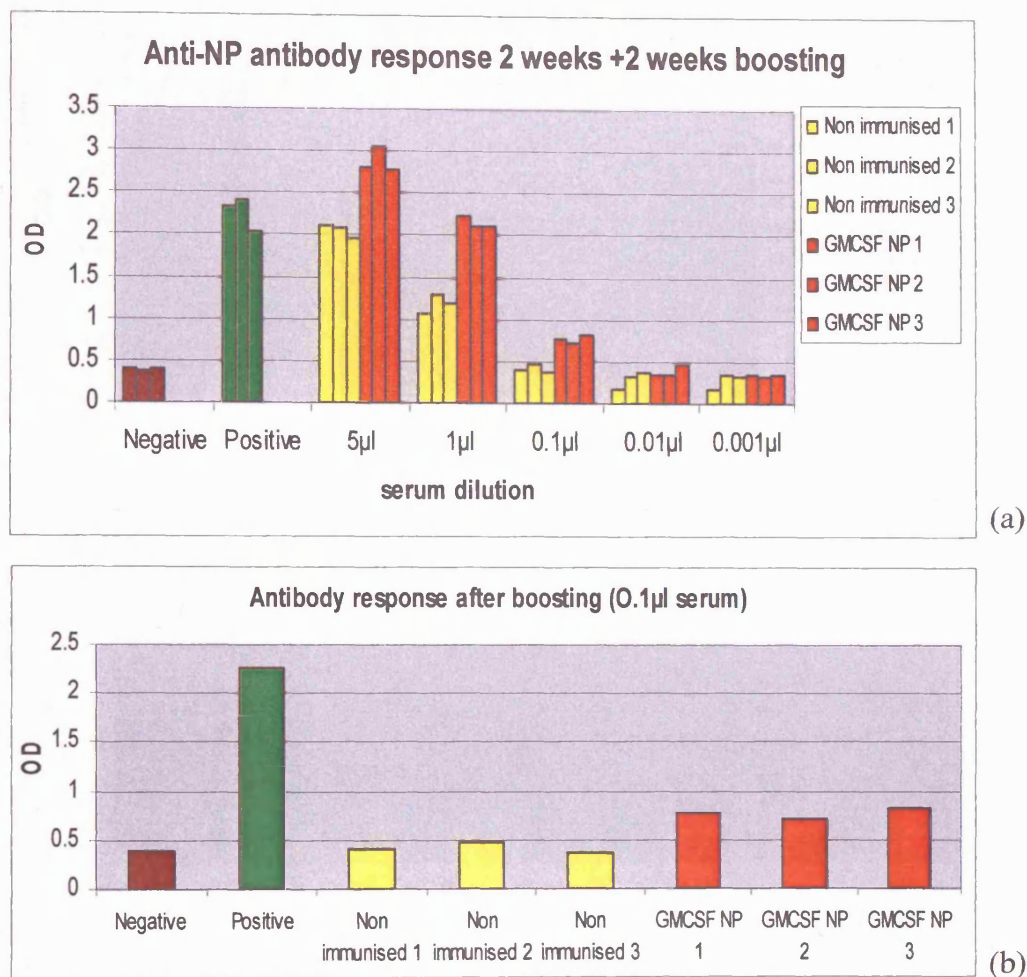


Figure 7.10: Anti-NP antibody response after 2 weeks boosting

Presence of anti-NP antibody in the serum of mice receiving a boosting injection two weeks after the primary immunisation with nothing (non immunised) or with 10^6 pfu of 17^{+47} -GMCSF-NP/vhs VP16⁻ (GMCSFNP). Serum was removed two weeks following the boost and various amounts were tested (a). The negative control is the value obtained by the addition of medium instead of serum and the positive control is the value obtained using the commercial anti-NP antibody instead of the serum sample. A comparison of the groups (b) was made at the amount of serum where non immunised mice give similar values to the negative control.

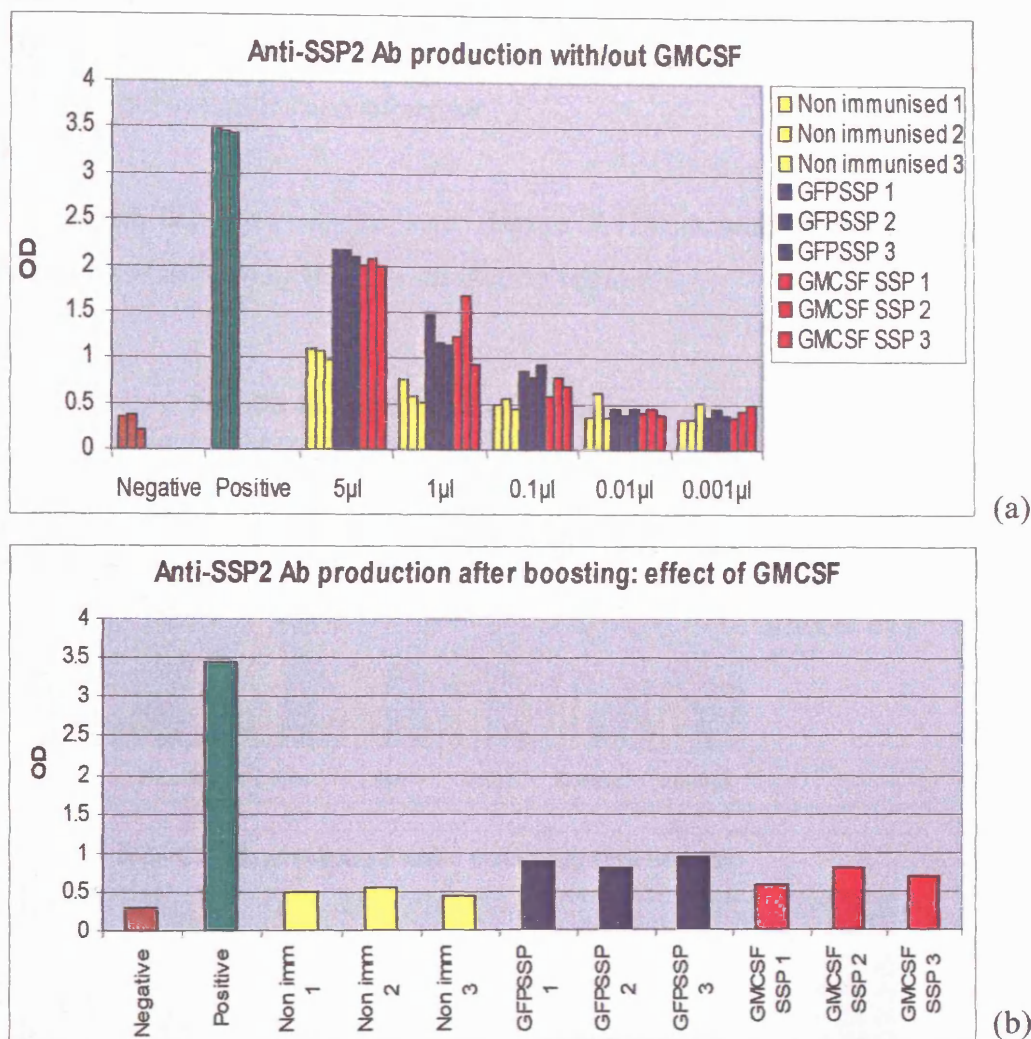


Figure 7.11: Anti-SSP antibody production after boosting: effect of GMCSF

Anti-SSP2 ELISA on serum samples from non immunised mice (yellow), or mice receiving a boost two weeks after immunisation with 10^6 pfu of 17^{+47} -GFP-SSP/vhsVP16⁻ (GFPSSP, blue) or neat 17^{+47} -GMCSF-SSP/vhs VP16⁻ (GMCSFSSP, pink). Serum was collected two weeks after boosting and tested at various dilutions and compared to the values obtained from using medium (negative, red) or F3B5anti-SSP2 (positive, green) instead of serum samples (a). The groups were compared more specifically at the serum dilution where non immunised mice give similar results to negative control (0.1µl) (b). The effect of GMCSF can be seen comparing the results from virus not containing the cytokine (blue) with those from immunisation with GMCSF virus (pink).

7.6.3 Effect of boosting on the induction of the antibody response to PyCS

Three mice per group were immunised with 10^6 pfu 17^{+47} GMCSF-CS/vhs VP16⁻ in one flank and footpad and boosted two weeks later with the same virus for a further 14 days before collection of serum.

Testing of serial dilutions of the sera (figure 7.12) showed a strong anti-CS antibody response following this immunisation regime.

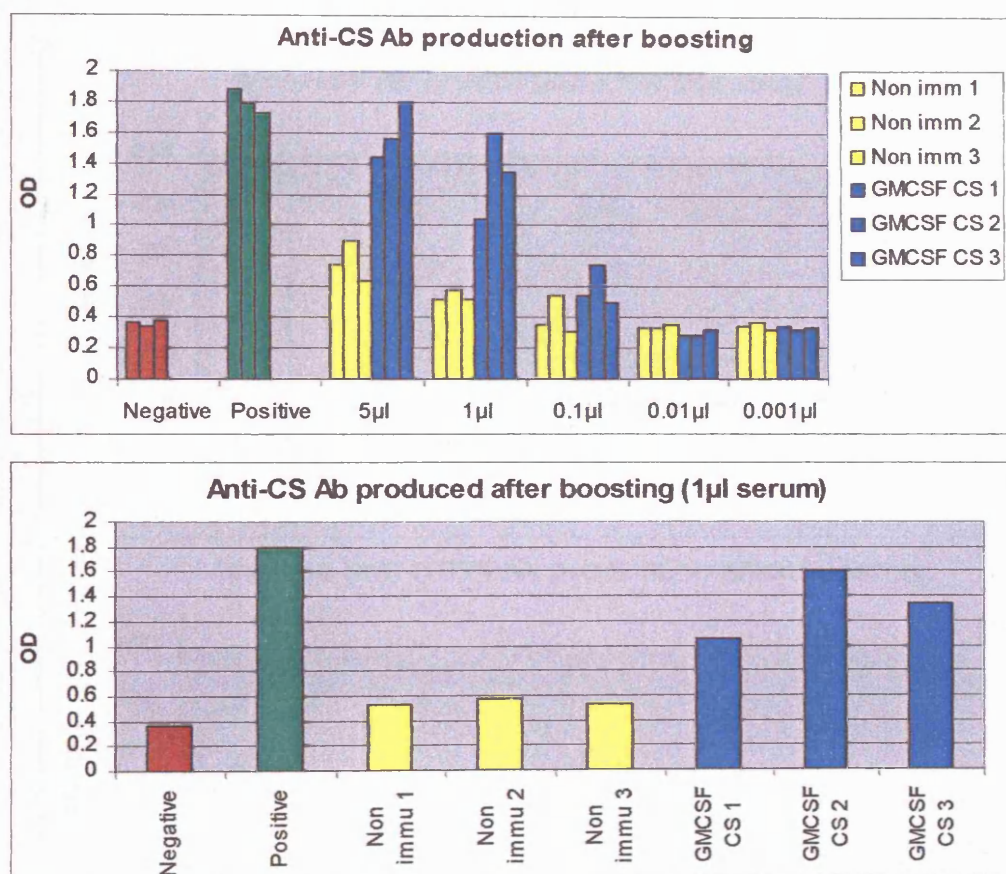


Figure 7.12: Anti-CS antibodies produced after boosting

Anti-CS ELISA on serum sample of non immunised mice (yellow) or mice receiving a primary immunisation with 17^{+47} GMCSF-CS/vhsVP16⁻ (GMCSFCS, blue) and a boost with the same virus two weeks later. Serum was removed 14 days following the boost, tested at various dilutions for anti-CS antibodies and compared to values obtained using medium (negative, red) or 93D anti-CS antibody (positive, green) instead of serum. The effect of the virus can be seen at every dilution comparing yellow and blue results and more specifically when using 1µl of serum where non immunised mice have similar values to the negative control.

7.6.4 Effect of boosting on the stimulation of the antibody response to HSV1

As for the recombinant protein, the response to HSV1 was expected to be stronger after boosting the immunisation. The serum samples from three mice immunised with $17^{+}47$ GMCSFNP/vhs VP16⁻, or non immunised, were tested in a neutralisation assay using $17^{+}47$ GMCSFNP/vhs VP16⁻ as input virus. As expected, virus was neutralised after boosting (figure 7.13), although, as no serial dilution of the serum was performed, the strength of the response can not be compared.

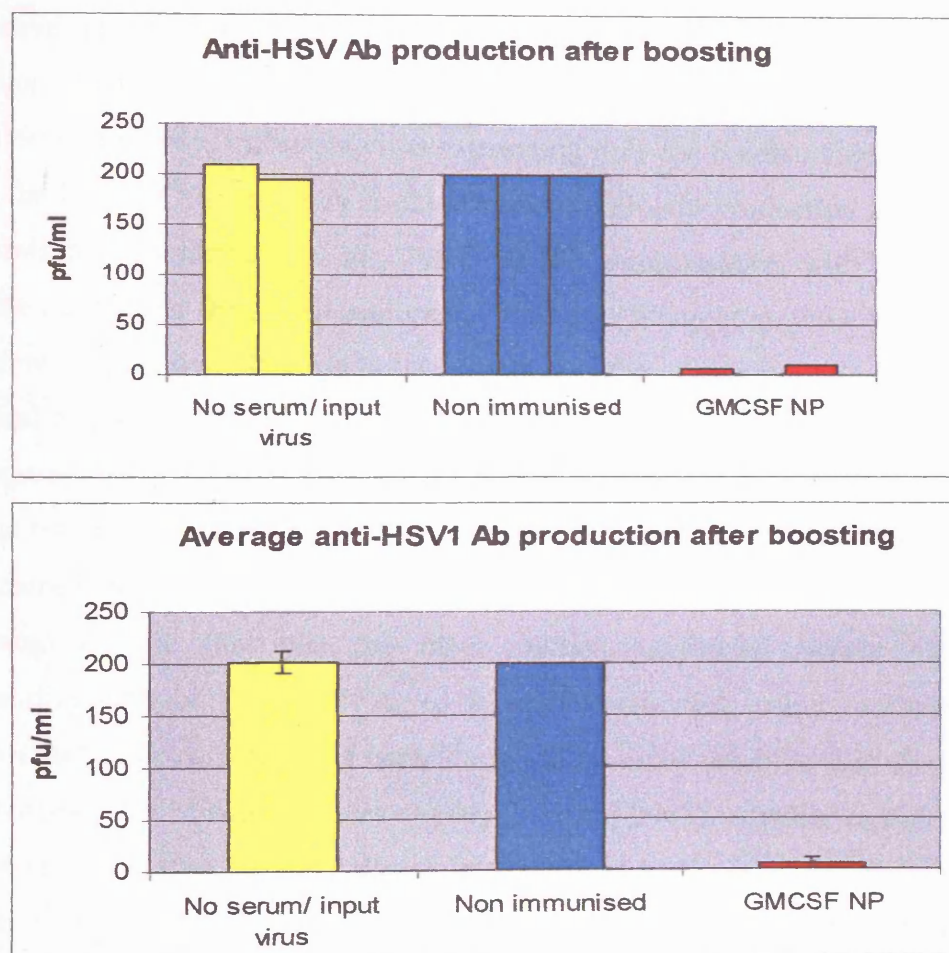


Figure 7.13: Anti-HSV1 antibodies after boosting

Neutralisation assay on serum samples from non immunised mice (blue) or mice receiving a first immunisation with $17^{+}47$ GMCSF-NP/vhsVP16⁻ (GMCSFNP) and a boost with the same virus two weeks later. Serum was removed 14 days following the boost and incubated with neat $17^{+}47$ GMCSF-NP/vhsVP16⁻ before plating on BHK cells for plaque assay. The serum samples can be compared to the number of plaques obtained by incubating the virus with medium instead of virus (yellow), this value corresponds to the amount of input virus.

7.7 Discussion

The results presented in this chapter have shown that in addition to cellular responses, HSV1 vectors can induce an antibody response both to the recombinant protein and to the virus.

It was surprising to see that antibodies against NP, CS, or SSP2 could only be detected after boosting. Previous studies have shown an antibody response to NP three weeks after single immunisation with a highly attenuated modified vaccinia Ankara (MVA) (Sutter et al., 1994) and that an immunisation with a replication-defective recombinant adenovirus expressing the PyCS protein induced, in addition to CD8 responses, high titres of anti-sporozoite antibodies (Rodrigues et al., 1997). Similarly, influenza virus expressing only the B cell epitope of PyCS or both the B and CD8 epitopes could stimulate antibody production after a single immunisation (Rodrigues et al., 1994) as did immunisation with a non linear peptide expressing the B cell epitope of PySSP2 (Wang et al., 1996). The results therefore suggest that HSV1 is not as efficient as the other viruses in stimulating a humoral response or alternatively that two weeks is an insufficient time for such responses to be efficiently stimulated. There is, however, an anti-HSV response, present two weeks after a single immunisation detectable in a neutralisation assay. That there is an anti-delivered protein antibody response present at two weeks is supported by the fact that the other studies mentioned mostly relied on immunofluorescence assays (IFA) to identify antibodies, using sporozoites or recombinant protein, which has been shown to be more sensitive than the ELISA test presented here using peptides (Sedegah et al., 2002). Another reason for the lack of response after the first immunisation might be the insufficient amount of protein expressed which could also explain the lack of enhancing effect from GMCSF expression. Experiments using recombinant adenovirus expressing PyCS showed that the response seen 12 days post-injection was dose related (Rodrigues et al., 1997), it is therefore possible that using a higher dose of HSV vector would result in detection of antibodies after the first immunisation.

A second injection was expected to increase the antibody response (diagram 7.1), and this turned out to be the case as robust antibody responses against the

recombinant proteins could then be detected. Being able to detect anti-NP, CS, or SSP2 antibodies after boosting is a sign that the strong neutralising response to the virus does not prevent the response to the recombinant proteins and suggests that the inability to detect antibodies after the primary immunisation is likely to be due to low levels of response rather than its absence. A 3-15 fold increase in the antibody response to NP has previously been reported after boosting a recombinant MVA immunisation (Sutter et al., 1994). Also, maximal responses have previously been obtained two weeks after boosting with vaccinia virus or MVA expressing PyCS (Gonzalez-Aseguinolaza et al., 2003). Less work has been performed using SSP2, but an increase in antibody responses have been detected in chimpanzees following three DNA immunisations and a boost of MVA expressing the same full length protein (Schneider et al., 2001). It could be that the initial low levels of antibodies produced against the recombinant proteins may give protection, but increasing this response as following boosting would be expected to be beneficial.

It would have been interesting to determine the isotype of antibodies produced both to HSV1 and to the proteins to confirm a Th1 type response. The fact that IFN γ is released as a cellular response to this immunisation is an indication of a Th1 mechanism, which seems to be appropriate to protect against malaria challenge (Wang et al., 1996). Anti-viral antibodies are only detected after neat virus injection and not after immunisation with infected DCs. This suggests that activation of B cells takes places via a CD4T cell mechanism rather than by direct contact of DC with B cells. Also, it indicates that the virus may infect other cell types that present viral antigens and activate B cells in a type 1 mechanism.

The advantages of being able to stimulate antibodies against PyCS or PySSP2 in particular, is still not clear. Passive transfer of anti-PyCS antibodies in Balb/c (Charoenvit et al., 1991b), and immunisation with adenoviruses expressing full length PyCS (Rodrigues et al., 1997) could induce both CTL and antibody responses upon s.c. immunisation and have led to protection, but numerous other studies have shown that induction of antibody responses in addition to CTL responses to malaria proteins is very often not necessary for protection (Khusmith

et al., 1991, Wang et al., 1996). It has been suggested that the reason that anti-SSP2 antibodies are not protective is that the protein is only released in close proximity of the cell to be infected, in which case antibodies only have a minimal opportunity to bind (Gantt et al., 2000). Nonetheless, having demonstrated production of antibodies is encouraging because the data presented here, especially for PyCS, reflects production of antibodies specific for the epitope that is known to be important for protection. When using full length genes, antibodies may in fact be raised against loci of the protein that do not play a role in protection, as demonstrated in a PyCS vaccine study carried out in monkeys (Charoenvit et al., 1991a). Expression of the B cell epitope of PyCS alone can confer a certain level of inhibition of the plasmodial liver stages (Rodrigues et al., 1994), and, in addition, the presence of both B and CTL epitopes of PyCS does not compromise the strength of responses of either, thus supporting the use of a full length gene and presentation of multiple antigens.

Being able to induce the highest possible antibody response may well be an advantage in the construction of a vaccine in order to achieve protection (Sedegah et al., 2000), and co-expression of GMCSF may help in doing this. The additional expression of GMCSF has previously been demonstrated to increase not only CTL responses but also antibody responses in mice immunised with plasmids encoding *P. yoelii* proteins and boosted with a recombinant poxvirus expressing the same protein (Hoffman & Doolan, 2000). Similarly, the level of antibodies has also been shown to be increased by GMCSF expression in monkeys by a live attenuated parainfluenza virus vaccine (Bukreyev et al., 2002). Addition of GMCSF in a plasmid based vaccine against PyCS has also been shown to increase the amount of antibody produced possibly in a CD4⁺T cells dependent fashion (Weiss et al., 1998). Despite the encouraging literature, in the previous chapter GMCSF co-expression from HSV-1 did not prove to have an enhancing effect on IFN γ responses. Therefore, not surprisingly, no advantage was seen of the expression of this cytokine in the antibody response against the recombinant proteins. From the data presented here, expression of GMCSF seems to have an effect mostly on the antiviral response after single immunisation, where the virus seems to be neutralised to a higher extent when GMCSF is expressed.

Contrary to a hepatitis B vaccine study where the addition of GMCSF led to antibody production after a single immunisation (Tarr et al., 1996), here GMCSF did not influence the level of response after boosting either. The reason for the effect of GMCSF may be due to the amount of protein produced and antigen presented and to the strength of the initial response, which seemed to be higher for the virus than the recombinant protein. The response obtained by boosting may be possibly enhanced further by increasing the time between the priming and the boosting (Bruna-Romero et al., 2001, Gonzalez-Aseguinolaza et al., 2003) and this might even produce enough antigen and cytokine to allow for GMCSF to have an effect.

Overall, the results obtained in this study, show specific induction of both branches of the immune system, with potential for malaria protection studies (Rodrigues et al., 1997). Despite the fact that antibodies to CS and SSP2 in particular have never been shown to be essential for protection, partially protected individuals in endemic areas show a response largely mediated by antibodies directed against blood stage antigens (Rogers et al., 2002). Therefore, having been able to show that it is possible to use this particular viral backbone to stimulate antibody responses is important, since it could be used to express other antigens, towards which an antibody response may be more important to give protection. MSP1 would be an appropriate antigen to include in such vaccine since antibodies against the C terminal of this protein have been associated with resistance to clinical malaria (Egan et al., 1996). Monoclonal antibodies against this protein are present in immune individuals and may account for the inhibition of invasion (Weiss et al., 1998) as well as inhibiting parasite growth in vitro (Egan et al., 1996). Alternatively, apical membrane antigen 1 (AMA1) would also be a good candidate considering that antibodies against this protein can inhibit erythrocyte invasion by merozoites (Hodder et al., 2001). Finally, MSP3 or glutamine-rich protein (GLURP) may be two more candidate proteins from the blood stage of the disease that would be appropriate to include in a vaccine able to stimulate antibodies since they can induce antibodies able to inhibit the growth of the parasite (Hodder et al., 2001, Oeuvray et al., 2000). Erythrocyte membrane protein 1 (EMP1) would also be an ideal candidate for such vaccine given its role in cyto-adherence of erythrocyte to endothelial cells, but the very high

in cyto-adherence of erythrocyte to endothelial cells, but the very high polymorphism of this protein and the presence of a large number of copies of this gene within each parasite might render antibodies against this protein ineffective (Richie & Saul, 2002).

Having identified that a boosting regime using neat virus is possibly the most suitable immunisation regime, it would have been appropriate to investigate the IFN γ response induced under these conditions. Extrapolating the results obtained by immunisation of one month followed by boost, it is possible to suggest that an improved cellular response might have been obtained too.

Chapter 8

General Discussion

HSV naturally infects dendritic cells (Coffin et al., 1998, Kruse et al., 2000, Mikloska et al., 2001, Salio et al., 1999) probably as an immune evasion mechanism since it prevents the cell from undergoing maturation (Kruse et al., 2000, Salio et al., 1999) and thus from presenting viral antigens to the immune system. HSV also expresses genes that disrupt the antigen presentation mechanism. This thesis has shown that HSV-1 with the virus host shutoff (vhs), and ICP47 proteins removed, and with the V422 mutation (Lam et al., 1996, Smiley & Duncan, 1997) in the VP16 gene can efficiently infect both human and mouse dendritic cells without compromising their ability to undergo maturation, allowing stimulation of immune responses *in vivo*.

The vhs protein had previously been shown to play a role in inhibiting maturation of HSV1 infected human dendritic cells (Samady et al., 2003). In this thesis it has been shown to have a similar function in mouse dendritic cells. Although removing only vhs from the viral backbone allowed for an improvement in the ability of the infected cells to mature, further attenuation of the virus by including the V422 transactivation terminating mutation in the C-terminal activation domain of the VP16 gene is necessary to allow complete maturation of the infected DCs. The ICP47 gene was also deleted so as to maximise antigen presentation. It was concluded that the 17⁺vhs⁻47⁻VP16⁻ backbone had the required characteristics for use as a vector for immunotherapy.

Injection of 17⁺vhs⁻47⁻VP16⁻ based viruses, or of dendritic cells infected with these viruses into the footpad of mice resulted in the trafficking of viral antigens to the draining lymph nodes, which also showed an increased CD11c⁺ cellularity. Immune responses are usually initiated in the lymph nodes where dendritic cells loaded with antigen drain from the infected site, and present peptides to naïve T cells within the node. Identification of the production of peroxidase in the LN was an indication of antigen arrival and presentation, and thus the probable initiation of an immune response. An increase of DC cellularity in the LN is also an indication of an ongoing immune response. This observation therefore implied that ex-vivo or in-vivo infected DCs retain their ability to mature and migrate *in vivo*, and to possibly induce an immune response. Injection of neat virus appeared to stimulate the highest level of CD11c⁺ cell accumulation.

Viruses with the 17⁺vhs⁻47⁻VP16⁻ backbone expressing influenza NP or plasmodium yoelii SSP2 or CS protein from the vhs site were constructed and used to investigate the possibility of using this vector to stimulate an immune response. Two weeks after subcutaneous immunisation in both flank and footpad, a CTL response to both the virus and recombinant protein could be detected, identified by IFN γ release from splenocytes. It appeared that splenocytes are activated by immunisation with non-transduced dendritic cells and that the NP peptide, but not the SSP2 or CS peptides are sufficient to prime these T cells in vitro. Despite these technical difficulties, the results obtained from the immunisation with the viruses expressing SSP2 or CS indicated the ability of this vector to induce a CTL response two weeks after immunisation with infected DCs or with neat virus.

In contrast to the results obtained from cell migration, immunisation with ex-vivo infected cells appeared to in general be slightly more efficient at generating a CTL response than using neat virus, although these differences were not significant. Injection of viral vectors is expected to induce a response both to the virus and to the delivered protein, and it is fundamental that a balance between the two occurs such that one does not block the other. Injection of infected DCs, but not of neat virus, one month after immunisation, was shown to stimulate an anti-viral response that was possibly too strong to allow a boosting regime, although this was necessary to ensure good antibody production to the delivered protein. The detection of a response to the protein after single (2 weeks) or repeated (1 month and 2 weeks) immunisation with neat virus is an indication that the anti-viral response does not mask the response directed against the delivered protein, showing that a balance has been achieved in the construction of the vectors.

Co-expression of cytokines, and of GMCSF in particular, has often been identified as a valuable means of enhancing the immune response (Weiss et al., 1998). In the case of GMCSF this is by stimulating DC recruitment. Contrary to the positive effect that GMCSF has when expressed from plasmid (Weiss et al., 1998) or adenovirus (Wang et al., 2002), coexpression of GMCSF from HSV-1

resulted in an increase in the level of response in only one case: injection of DCs infected with virus encoding NP. The effect of GMCSF on the ability of a viral vector to induce an immune response would not be expected to be determined by the particular recombinant protein expressed. Since GMCSF did not show any enhancing effect from injection of DCs infected with viruses expressing PySSP2, or from immunisation with neat virus expressing NP or PySSP2, it was concluded that, in general, GMCSF has no effect on the strength of the response induced when expressed from the HSV vectors. Despite the inability of GMCSF to enhance the immune response in this case, GMCSF was still shown to have a beneficial effect by improving reproducibility of the response induced when injecting neat virus, regardless of the antigen expressed. The amount of GMCSF released by the ex-vivo or in-vivo infected cells is important (Miller et al., 2002). Possibly, in these experiments, inefficient GMCSF was released to stimulate an increase in the response, although sufficient amounts were present to ensure a minimal but constant flow of DC recruitment, possibly accounting for the reproducible response.

Malaria is a multistage pathology that comprises both intracellular and extracellular phases. Vaccines in development are using vectors that express antigens (full length or peptides) from various stages of the disease (Ockenhouse et al., 1998, Shi et al., 1999, Tine et al., 1996), indicating that both cellular and humoral responses are thought to be important. Ideally, a vaccine vector should be able to stimulate both branches of the immune system allowing application to a variety of pathologies independent of the type of protective response required. The 17⁺ vhs⁻ 47⁻ VP16⁻ based HSV1 vectors expressing GMCSF together with NP, PySSP2 or PyCS, were shown to stimulate production of antibodies against the vector and the recombinant proteins. Interestingly, virus neutralising antibodies are only detected after neat virus injection and not after immunisation with infected DCs indicating that the virus, in vivo, probably infects different cell types (eg. macrophages) that trigger a mechanism that is more efficient than ex-vivo infected DCs at stimulating antibody responses.

That a boosting regime was necessary to detect antibodies against the recombinant proteins implied that a single immunisation only produced relatively low levels of

antibody. A boosting regime, however, resulted in a strong antibody response against the recombinant proteins that was not masked by the response against HSV.

Overall, this study has shown that immunisation with neat 17⁺ vhs⁻47⁻VP16⁻ based viruses expressing GMCSF together with a delivered protein can stimulate a Th1 type response inducing production of CTL (IFN γ release) and antibodies against the delivered protein, in this case from malaria. A prime-boost regime appears to allow a better humoral response, and may also be appropriate for a maximal cellular response, although cellular responses following two weeks priming and two weeks boosting need to be determined. The finding that injection of neat virus is as efficient as immunisation with infected DCs provides a potential advantage in vaccine development as this reduces the complexity of the treatment.

The results are promising seen that experiments aimed at achieving protection against malaria challenge might similarly be performed in mice. However, more work should be undertaken using the HSV-1 vector, particularly investigating its ability to induce similar levels of immune responses when expressing multiple malaria proteins together. LSA1 is a good candidate since expressed by liver stage parasites and studies on naturally exposed populations have revealed a connection between protection and immune responses against this protein (Kurtis et al., 2001). MSP1 is another antigen of interest since responses, in particular antibodies against it would block entry into RBC. The use of multiple antigens is now recognised as one of the best strategies in malaria vaccine development, particularly due to the high antigenic polymorphism of malaria protein and as it has already been shown that this does not induce inhibiting competition between antigens (Saul et al., 1999). Ring infected erythrocyte surface antigen (RESA) and apical membrane antigen are two more antigens that should be included in a multiantigen vaccine.

Glossary

μl:	microliter
aa:	amino acid
AAV :	Adeno associated virus
Ab :	Antibody
ADCC:	antibody dependent cellular cytotoxicity
AdPyCS:	Adenovirus expressing PyCS
AEC:	3-amino-9-ethyl-carbazole
Ag :	Antigen
AMA:	Apical membrane antigen
APC :	Antigen presenting cell
APL :	Altered peptide ligand
ARE:	AU-rich elements
BHK:	Baby hamster kidney cells
BM :	Bone marrow
BMDC:	Bone marrow dendritic cell
bp:	Base pair
BSA:	Bovine serum albumin
CAR:	Coxsackievirus and adenovirus receptor
CCR:	CC receptor molecule
CD:	Cluster determinant
CEA:	Carcinoembryonic antigen
CIAP:	Calf intestinal alkaline phosphatase
CLP :	Common lymphoid precursor
CMP :	Common myeloid precursor
CMV:	Cytomegalovirus
CNS:	Central nervous system
CPE:	complete cytopathic effect
CS :	Circumsporozoite surface protein
CTL:	Cytotoxic T lymphocyte
DAB:	Biaminobenzidine
DC :	Dendritic cell
DEPC:	Diethylpyrocarbonate
DISC:	Disabled infectious single cycle
DMED:	Dulbecco's Modified Eagle medium
DMF:	N,N-dimethylformamide
DMSO:	Dimethylsulphoxide
DNA:	Deoxyribonucleic acid
DRG:	Dorsal root ganglia
E:	Early genes
EBV:	Epstein-Barr virus
ELISA:	Enzyme linked immunosorbent assay
ELISPOT:	Enzyme linked immunospot assay
EMP:	Erythrocyte membrane protein
FACS:	Fluorescence activated cell sorting
FCS:	Foetal calf serum
f.p:	Footpad
FP:	Fowlpox

g:	gram
GFP:	Green fluorescent protein
GLURP:	Glutamine-rich protein
GMCSF:	Granulocyte monocyte colony stimulating factor
HbsAg:	Hepatitis B surface antigen
HBSS:	Hank's balanced salt solution
HCF:	Host cell factor
HEV:	High endothelial venules
HHV:	Human herpes virus
HIV:	Human immunodeficiency virus
HMBA:	Hexamethylene bisacetamide
HRP:	Horse radish peroxidase
HSV:	Herpes simplex virus
HVEM:	Herpes virus entry mediator
ICP:	Infectious cell protein
i.d.:	intradermal
IE:	Immediate early
IFA :	immunofluorescence assay
IFN:	Interferon
Ig:	Immunoglobulin
IL :	Interleukin
i.m.:	intramuscular
IRE:	Interferon response element
IRES:	Internal ribosome entry site
IRF:	Interferon regulatory factor
ISG:	Interferon stimulated gene
ITR:	Inverted terminal repeat
i.v:	Intravenous
Kb:	Kilobase
kDa:	KiloDalton
L:	Late genes
LAT:	Latency associated transcripts
LB:	Luria Bertani
LCMV:	Lymphocytic choriomeningitic virus
LN :	Lymph node
LPS :	Lypopolysaccharide
LSA:	Liver stage antigen
LTR :	Long terminal repeat
M:	Molar
MAC:	Membrane attack complex
MAP:	Multiple antigen peptide
MDC:	Macrophage derived chemokine
ME:	Multiple epitope
mGMCSF:	mouse GMCSF
MHC:	Major histocompatibility complex
MIP:	Macrophage inflammatory protein
ml:	millilitre
MLV:	Murine leukaemia virus
MMLV:	Moloney murine leukaemia virus
MOI:	Multiplicity of infection

MSP:	Merozoite surface protein
MVA:	Modified vaccinia Ankara
NK:	Natural killer
NP:	Nucleoprotein
NYVAC:	New York vaccinia strain
OAS:	Oligo-adenylate synthase
OD:	Optical density
PAGE:	Polyacrylamide gel electrophoresis
PAMP:	Pathogen-associated molecular patterns
Pb:	Plasmodium <i>berghei</i>
PBMC:	Peripheral blood mononuclear cells
PBS:	Phosphate buffer solution
PCR:	Polymerase chain reaction
PfEMP1	Plasmodium falciparum erythrocyte membrane protein 1
PMA:	Phorbol 12-myristate 13-acetate
PML:	Premyelocytic leukaemia
PP:	Protein phosphatase
Py:	Plasmodium <i>yoelii</i>
PyHep:	Plasmodium <i>yoelii</i> hepatocyte erythrocyte protein
RBC:	Red blood cell
RESA:	Ring infected erythrocyte surface antigen
RFP:	Red fluorescent protein
RNA:	Ribonucleic acid
ROI:	Reactive oxygen intermediate
RSV:	Rous sarcoma virus
RT PCR:	Reverse transcription polymerase chain reaction
s.c:	Subcutaneous
SDS:	Sodium dodecyl sulphate
SERA:	Serine repeat antigen
SFM:	Serum free medium
SIN:	Sindbis virus
SIV:	Simian immunodeficiency virus
SSP2:	Sporozoite surface protein 2
STAT:	Signal transducer and activator
TAP:	Transport associated protein
TARC:	Thymus and activation regulated chemokine
TGF:	Tumour growth factor
Th:	T helper cell
TLR:	Toll like receptor
TNF:	Tumour necrosis factor
TRAP:	Thrombospondin associated protein
Treg:	Regulatory T cell
Ty-VLP:	Ty-virus like particle
UL:	Unique long
US:	Unique short
Vhs:	Virion host shutoff protein
VIC:	VP16 induced complex
VLP:	Virus like protein
VP16:	Virion protein 16
VZV:	Varicella zoster virus

Chapter 9

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